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# The Effect Of Dietary Folate On Tissue Folate Status

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**THE EFFECT OF DIETARY FOLATE ON TISSUE FOLATE STATUS**

by

**GHADA AOUN**

**THESIS**

Submitted to the graduate school

of Wayne State University

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**MASTER OF SCIENCE**

2015

Major: Nutrition Food And Science

Approved By:

---

Advisor

Date

## **DEDICATION**

I would like to dedicate my work to my loving, encouraging, and supportive husband; and my lovely kids.

## **ACKNOWLEDGMENTS**

I would first and foremost like to deeply thank my Advisor Dr. Diane Cabelof who gave me the opportunity to pursue a Masters under her supervision. Thank you for your support and encouragement. I am so grateful for your patience. You have been given me an endless time and support. You are a great mentor. I also want to acknowledge my committee members; Dr. Heydari and Dr. Zhou for their support. I would like to thank my friends especially Honghzi Ma for her help and thoughtful insight throughout my project, and to my friends Aqila, Khadijah, Catherine, and Cristine for their encouragement and help, and I would like to thank Mei Jung York for her support.

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## CHAPTER 1: INTRODUCTION

### Nutrition, Folate, and Disease

Nutrition is a main health concern from the moment of conception to each stage of advanced age. As a field, nutrition involves applying principles from a range of fields including molecular biochemistry and statistical epidemiology to areas of interest such as energy metabolism, population, health, and aging. A primary objective of nutrition research is evaluating how a specific diet or nutrient affects the transcription and expression of genes that control cellular processes throughout the human body; likewise, research is also geared towards understanding the influence of individual genetic variation on diet and nutrition (1).

My focus will be investigating the effects of dietary folate on tissue folate status, the impact of folate deficiency on tissues and cellular pathways, and the distribution of folates throughout the entire body. Many research studies investigate only serum folate levels and extrapolate that information to the tissue level. We are interested in evaluating how different tissues actually respond to a lack of dietary folate, and how well these values correlate to folate levels in serum.

Folate is a general term for a number of folic acid derivatives that have the biological activity of folic acid (pteroylglutamic acid). Folate is made up of three parts. 2-amino-4-hydroxypteridine, also called aromatic pteridine ring, is conjugated by a methylene group ( $\text{CH}_2$ ) to para-aminobenzoic acid (PABA) to form pteronic acid (2). The carboxyl group of PABA is peptide-bound to the amino group of glutamic acid to form folate, producing pteroylmonoglutamate. Folate vitamers differ in the oxidation state of the pteridine ring and substitution on the N5 and N10 nitrogen atom as seen in figure 1.1 (3). Folates are found naturally in food, with the primary dietary sources of folate being green vegetables such as broccoli, spinach, asparagus, okra, and turnip greens, as well as fruits (such as strawberries and

oranges), legumes (especially lima, pinto, and kidney beans), and liver (4). Naturally occurring folate in foods exists as reduced polyglutamates and may contain up to nine glutamic acid residues. While the reduced polyglutamates are found in plant foods and animals, in fortified foods and supplements, the fully oxidized form (folic acid) exists primarily as pteroylmonoglutamate, the most stable and oxidized form of folic acid (5).

Folate and its biological role were first identified by researcher Lucy Wills in 1931. She found that folate was needed to prevent anemia during pregnancy. Wills demonstrated that yeast extract was effective against anemia, and in the later 1930s, folate was extracted from yeast (6). Folate has a central role in biological methylation and nucleotide synthesis; therefore, a folate deficient diet has been observed to alter and diminish DNA stability, and to modulate carcinogenesis (7). The role of folate in DNA synthesis and repair has made this nutrient of high interest in aging research. Most folate research has focused on folate deficiency and its relationship to impaired cell division and multiple disease outcomes. However, this complex biochemical pathway is affected by nutritional and genetic factors, and we suggest a better understanding of these influences will aid in understanding how folate affects general human health.

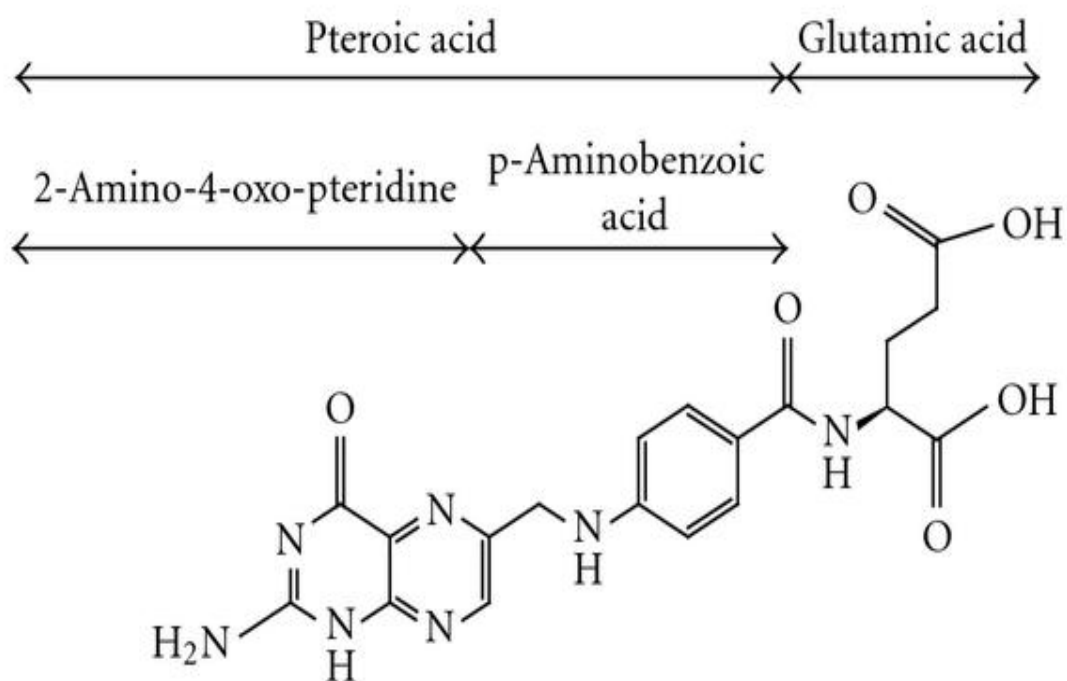


Figure 1: Chemical Structure of Folic Acid (8)

The three parts of the folate structure include: 2-amino-4-oxo-pteridine, p-aminobenzoic acid, and glutamic acid.

## **A. Folate Nutrition**

### **1. Bioavailability of Folate:**

Folate refers to a group of water-soluble vitamins that occur naturally in foods as pteroylglutamic acid (PGA). The most common natural folates are polyglutamates: tetrahydrofolic acid, 5-methyl tetrahydrofolic acid, and 5-formyl tetrahydrofolic acid. The biologically active form, tetrahydrofolate, is considered more stable than dietary folate. THFA is produced by the two-step reduction of folate using the enzyme dihydrofolate reductase (2). Folic acid is about 85% bioavailable when consumed with food. However, folate bioavailability from foods is reduced by as much as 25-35% once the polyglutamate chain has been removed by the intestinal conjugase (9).

Food fortification can increase the amount of folic acid in the diet. The Recommended Dietary Allowances (RDA) for adults for folate is 400 µg dietary folate equivalents (DFA) per day, amounts generally found in ready-to-eat cereals (10). Dietary Folate Equivalent is a term used to express the RDAs for folate in micro grams (µg). One DFE is equal to one µg of food folate, which is equal to 0.6 µg of folic acid from supplement and fortified food consumed with food, which is equal to 0.5 µg consumed without food (10).

Diets with adequate sources of folate may reduce a woman's risk of having a child with a neural tube defect. According to the Center for Disease control and Prevention guidelines in US, women in the childbearing age recommended their intake of folic acid to 0.4 mg per day to protect against neural tube defects. These guidelines are similar to those of Centers for Disease Control and Prevention and the Department of Health in Canada and Britain (11).

## 2. Absorption and Transport

In humans, the small intestine is capable of absorbing folate in the mono- or di-glutamated forms, which occur mostly in the jejunum of the small intestine. In order to absorb the polyglutamate form of 5-methyltetrahydrofolate, dietary folate is hydrolyzed by intestinal brush border gamma-glutamyl hydrolyze (also called conjugase or folylpolyglutamate). The monoglutamate form of folate is absorbed by a pH dependent carrier-mediated process (pH 6.3) after cleavage, and can then disperse across the cell membrane (12). If folate intake is increased by supplementation, the folate diffusion process becomes more prevalent. Natural folates are labile compounds that can be easily lost during food preparation and consumption. The 5-methyldihydrofolate is rapidly degraded under acidic conditions, while 5-methylTHF is more stable. Ascorbic acid is a critical factor in reducing 5-methylDHF back to more stable 5-methylTHF before absorption (12).

There are two major sources of folate within the intestine. The first one is dietary folate, which is absorbed throughout the small intestine. The second source is through the bacteria that are synthesized by the normal microflora of the large intestine. However, various conditions can affect the population of micro flora such as the consumption of alcohol, antibiotics, and cancer drugs, which interfere with the micro-flora's ability to synthesize folate. Bacterially synthesized folate can be absorbed across the large intestine and incorporated into the liver and other tissues (12).

## **B. Function and Role of Folate**

Folate plays a role in both oxidation reactions and the transfer of one-carbon units from one compound to another. The central folate acceptor molecule in the one-carbon cycle is a polyglutamyl form of tetrahydrofolate (THF). This polyglutamyl chain is required for folates to perform their biological functions and to retain folate in the cell (3). Tetrahydrofolate works in the body as a coenzyme to accept one-carbon groups. The derivatives of THF participate in many different metabolic pathways. As seen in figure 1.2, 5-methyl THF is the methyl donor for the conversion of dUMP (deoxyuridine monophosphate or deoxyuridylate) to dTMP (deoxythymidine monophosphate or deoxythymidylate) a reaction catalyzed by thymidylate synthase (TS). Tetrahydrofolate accepts single carbon units (methyl group) from serine, converts serine to glycine, and forms 5-10-methylenetetrahydrofolate. Furthermore, folate coenzymes are involved in other amino acid inter conversion such as conversion of histidine to glutamic acid and homocysteine to methionine (15).

Folate has two major biochemical functions. One is mediating the transfer of one-carbon units involved in biological methylation reactions. The other is nucleotide biosynthesis, acting as the methyl donor for thymidylate synthase to create deoxythymidine-5-monophosphate (dTMP) (pyrimidine synthesis), as well as the purine synthesis which requires 10-formyl-THF for the C2 carbons of the purine ring, the remethylation of homocysteine to methionine, which requires 5-methyl-THF, and the formation of the primary methylating agent (S-adenosylmethionine) as seen in figure 1.2 (16). Since folate is a mediator of one carbon metabolism, it plays a crucial role in the synthesis of DNA and in the reproduction of cells in the fetus. Moreover, folate helps in maintaining the nervous system, functions of the intestinal tract, and is also essential in the formation of red blood cells. (17).

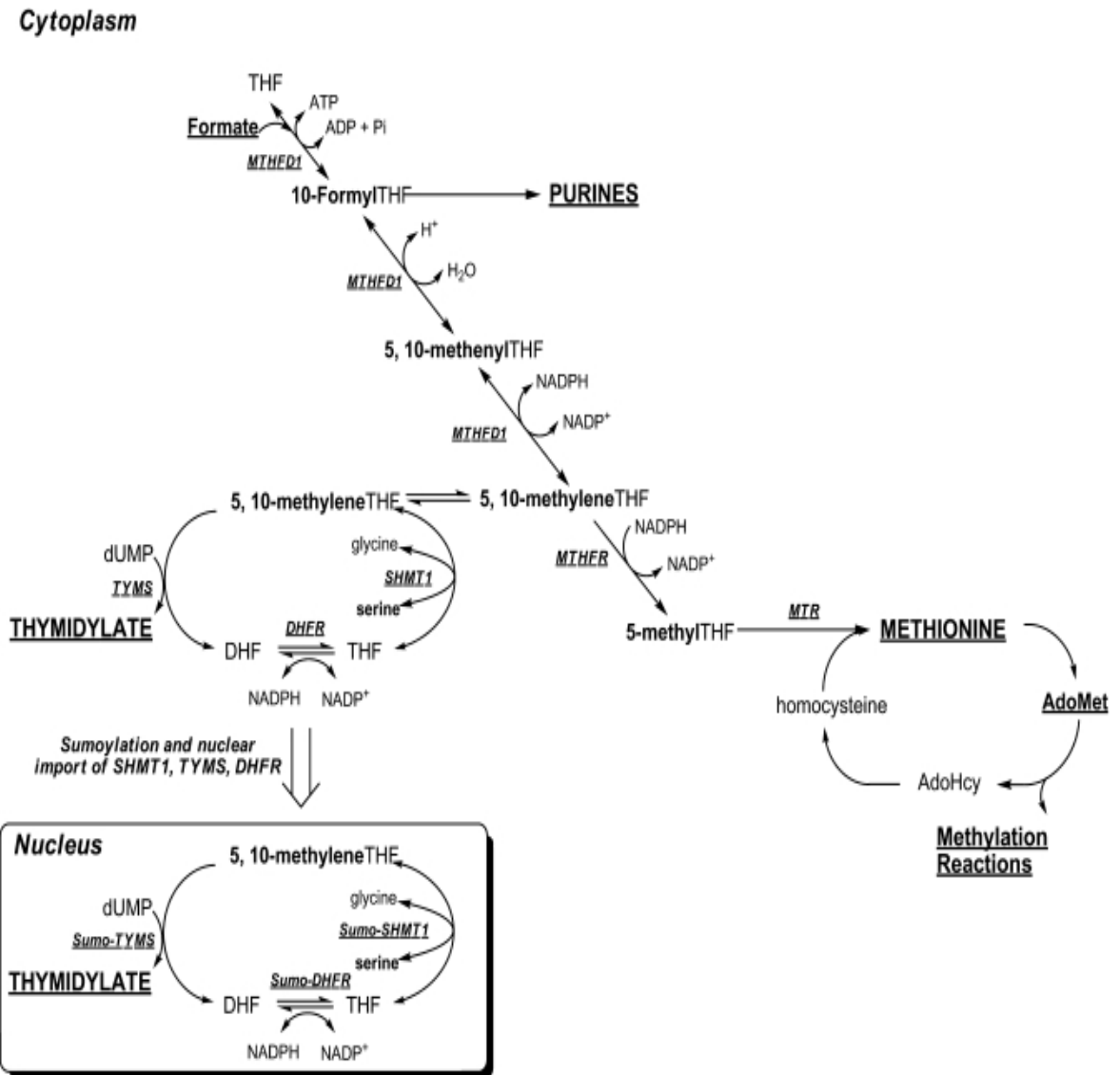


Figure 2: Folate Metabolism Pathway (18)

### **C. Folate Deficiency and Adverse Effects:**

Research over recent decades has shown that folate deficiency is an important public health concern because of its contribution to both congenital malformations and development of chronic disorders, including congenital neural tube defects (NTD) and megaloblastic anemia. Folate deficiency can also disrupt epigenetic changes in gene expression, such as DNA methylation, which can lead to diseases such as colon cancer (2).

#### **1. Folate deficiency and NTDs:**

Congenital neural tube defects (NTDs), specifically anencephaly and spina bifida, are birth defects that occur early in embryonic development, and are the most frequently occurring congenital abnormalities of the central nervous system (19). The spinal cord and the brain develop from the neural tube two weeks post-conception. Sometimes, however, the brain begins to develop outside the skull, which results in the malformation of the brain called anencephaly, or spina bifida (the malformation of spinal cord). Clinical research studies have correlated folate deficiency to NTD incidence, and increased folate intake with lower rates of NTD (19).

Research has also shown that serum/plasma folate concentrations increase in response to greater folic acid intake; that is, the level of folate in the blood can be increased through diet and supplementation. Given the clear relationship between NTDs and folate deficiency, United States mandated a fortification program for women with a history of NTD-affected pregnancy who plan to become pregnant, should provide 100 gram enriched cereal grain product of folic acid per day (20). However, even after these recommendations were issued, a reduction in NTDs did not follow. Therefore, folic acid food fortification was mandated in the United States, Canada and several other countries (20). In 1998, the US Department of Health and Human



Services ordered food fortification with folic acid for all cereal grain products. Following this decision, national folate status improved, and the U.S. saw a 26% reduction in the prevalence of NTDs (21). Canada similarly mandated a flour fortification with folic acid, and saw a 19%-55% decrease in NTDs, and improvement in overall folate status (21). Therefore, fortification is one of the effective methods to avoid nutritional deficiencies.

The United States and Canadian governments continue to mandate folate enrichment of food, and strongly encourage high folic acid intake for women of childbearing age. Currently, both countries recommend all women planning to get pregnant consume 400 µg/d of folic acid through diet and supplementation to prevent NTD, up from the 400 µg/daily recommended in 1992 (20). The Centers for Disease Control and Prevention further encourage women who have a history of an NTD-affected pregnancy to begin consuming 400 to 500 µg of folic acid daily as soon as they decide to become pregnant (22).

## 2. Megaloblastic Macrocytic Anemia and Folate Deficiency

Megaloblastic anemia is an anemia in which red blood cells (RBCs) become abnormally large, impairing their function. In addition to the cells being large, the inner contents of each cell are not completely formed. As a result of these changes, RBCs cannot carry oxygen or travel through capillaries (23). Individuals with megaloblastic anemia may exhibit symptoms such as fatigue, irritability, weakness, difficulty concentrating, palpitations, shortness of breath, and decrease in appetite (2)

This abnormality of megaloblastic anemia results from low plasma folate. During folate deficiency, DNA damage prevents division and maturation of RBCs, causing the bone marrow to produce fewer cells (23). Further, the concentration of homocysteine increases and leads to megaloblastic changes, with rapidly dividing cells characterized by impaired DNA synthesis.

Bone marrow cells can become megaloblastic and take on the oval cell shape after as little as three to four months of low folate intake (23). Megaloblastic anemia related to folate deficiency is relatively common in United States.

### 3. Epigenetic Change and Cancer

Although the exact mechanisms are not yet fully understood, epidemiological studies suggest that folate may play a role in preventing a variety of cancers by affecting epigenetic change. Epigenetic change alters gene expression and can cause loss of DNA function, which can lead to the development of cancerous cells. For example, DNA methylation depends upon the availability of methyl groups from S-adenosylmethionine (SAM) (24). Folate plays a crucial role in one-carbon metabolism related to DNA methylation: as seen in figure 1.3, when dietary folate is consumed, the absorbed folate is metabolized to 5-methyltetrahydrofolate (5-MTHF) (monoglutamate form) in the intestine, which then must be polyglutamated for cellular retention and one-carbon coenzyme function. 5-MeTHF must be converted to tetrahydrofolate by methionine synthase reaction. The methyl groups enable the remethylation of homocysteine to methionine, providing the substrate for the latter, which in turn produces SAM. A similar process occurs when synthetic folate is consumed: after being reduced to dihydrofolate (DHF) by dihydrofolate reductase, and then to THF to enter the folate pathway, it is converted to 5,10-methyleneTHF by vitamin B-6-dependent enzyme called serine hydroxymethyltransferase, and then irreversibly reduced to 5-MeTHF by methylene tetrahydrofolate reductase (MTHFR). The 5-MeTHF from synthetic folate can then be utilized in the same methionine synthase reaction as the 5-MeTHF sourced from dietary folate (24).

When folate is in inadequate supply, the reactions dependent upon the methyl groups produced by the breakdown of folate are inhibited. As a result, SAM concentrations are affected, slowing down the DNA methylation process. High global DNA methylation is associated with reduced risk of cancer.

Therefore, consuming adequate amounts of folate through diet and supplementation appears to be crucial to preventing the kinds of epigenetic change associated with various cancers.

We can see the impact of folate consumption on cancer development by looking at one of the most common cancers, colon cancer. Studies have suggested that DNA methylation is an indicator for colon cancer. As demonstrated above, folate availability affects the DNA methylation process. According to observational studies, the inverse association between folic acid consumption and colon cancer risk was greatest in women with a family history of the disease. Data confirmed that for this population, colon cancer was reduced by 52% in women who consumed more than 400  $\mu\text{g}/\text{d}$  compared with women who consumed less than 200  $\mu\text{g}/\text{d}$  (25). Regular dietary supplementation of folic acid for more than five years may diminish the risk of colon cancer associated with family history of this cancer (25). Since folate metabolism affects a variety of epigenetic changes, including DNA synthesis, genomic stability, cell division, methylation, and gene expression, researching folate metabolism may be important to cancer prevention and treatment.

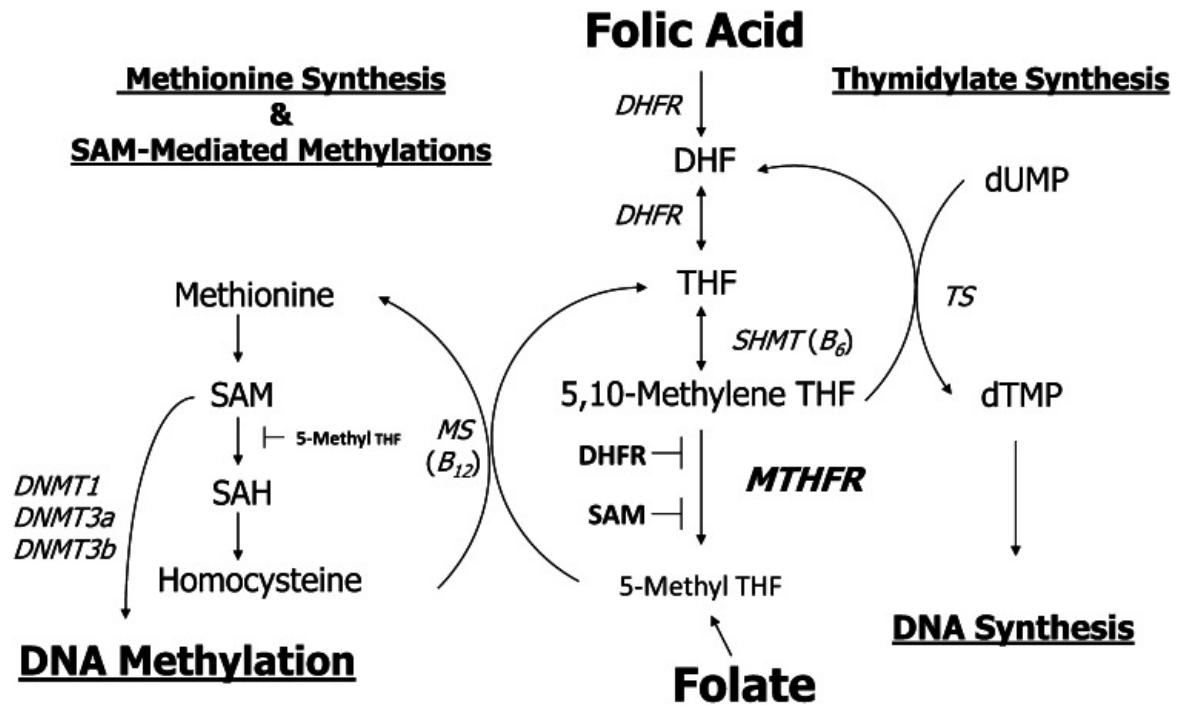
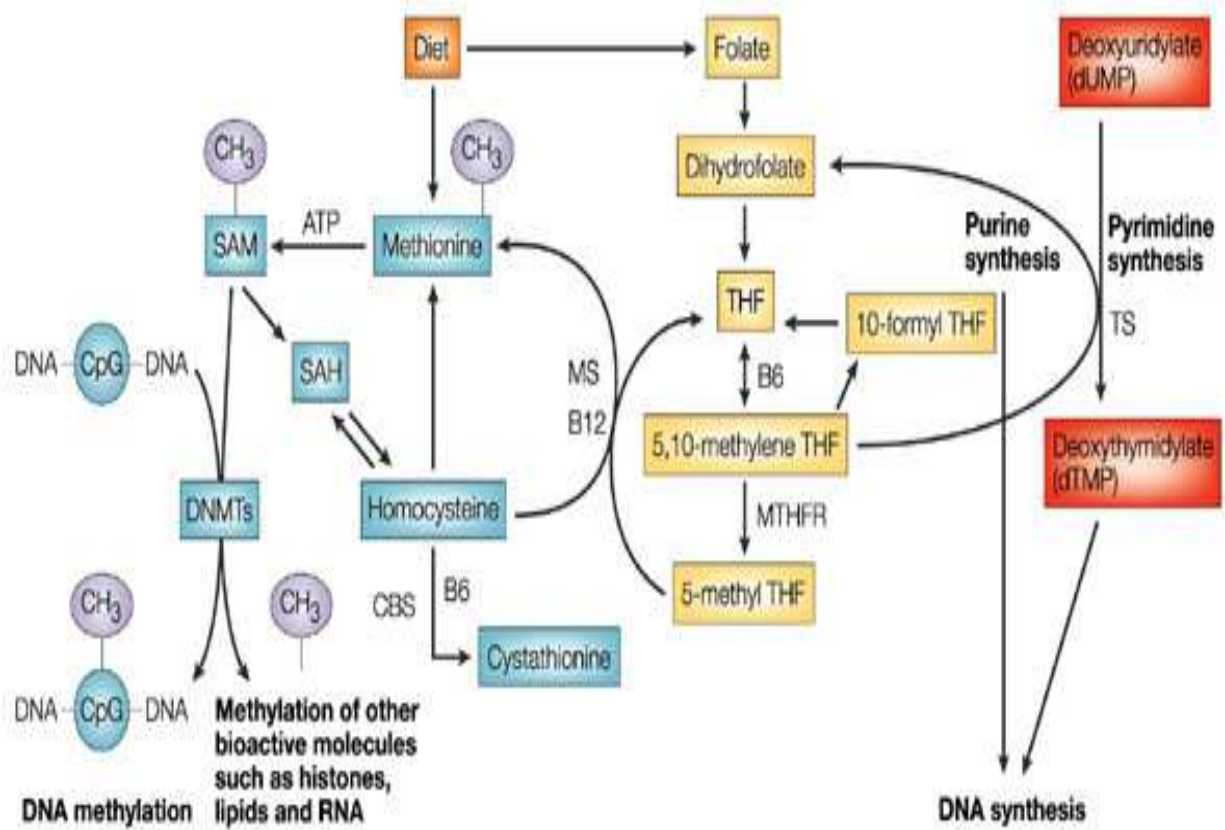


Figure 3: Folic acid metabolism and the process of DNA methylation (24).

#### **D. Folate in One-Carbon Metabolism:**

As discussed in the previous section, research has shown how important folate is for disease prevention. At the same time, basic bodily health also depends heavily on folate status: folate is an essential vitamin that is involved in cellular and metabolic functions such as DNA synthesis and cell division. Therefore, it is important to understand how folic acid status alters the one-carbon metabolism.

As seen in Figure 1.3, methylation reactions that utilize S-adenosylmethionine (SAM), as well as folate-dependent synthesis of thymidine and purines are needed for DNA synthesis and repair. THF, one of the folate derivatives, yields three one-carbon substituted derivatives: 5-methyl THF, which is required for methionine synthesis; 5,10-methylene THF, which is essential for the synthesis of deoxythymidylate, a pyrimidine component of DNA; and 10-formyl THF, which is a cofactor for purine synthesis. Methylation reactions such as the use of S-adenosylmethionine (SAM), which is converted to S-adenosylhomocysteine (SAH), and also the synthesis of thymidine and purines, are needed for DNA synthesis and repair (24). Vitamin B12 works as an essential co-enzyme in the transfer of the methyl group from 5-methyl THF to homocysteine, yielding methionine. In addition to protein synthesis, methionine serves as a methyl group donor through conversion to SAM, a key methylation agent that is involved in methyltransferase reactions. (3)



Nature Reviews | Cancer

Figure 4: Folate Metabolic Pathways (26)

As figure 1.4 shows, as we mentioned before, folate is involved in essential functions of cell metabolism such as methylation and synthesis of nucleotides, and some amino acids. The folate influences the thymidine monophosphate (dTMP), and the purine nucleotides synthesis consequently affects DNA synthesis and repair. Since folate is involved in DNA methylation, it therefore affects gene expression (27).

#### **E. Genes related to Folate metabolic pathways:**

Because of the roles folate plays in DNA synthesis and growth, it is important to examine the enzymes that determine the accessibility of folate in these folate-dependent pathways. Any variation affecting the functioning of the enzyme could negatively impact folate metabolism, and subsequently, health.

The MTHFR enzyme, a central enzyme in folate metabolism, plays a crucial role in directing the folate pool toward the remethylation of homocysteine to methionine. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as methyl donor for the remethylation of homocysteine to methionine and the precursor of S-adenosylmethionine. Variation in MTHFR could reduce the activity of the enzyme, and hence will affect the availability of folate and lead to altered methylation patterns (18). Low activity of MTHFR leads to an inadequate remethylation of homocysteine to methionine and to a decrease production of S-adenosyl-methionine (SAM). Any mutation in the MTHFR gene will disturb the pathway of folate and reduces or affects the synthesis of DNA (28).

Gamma-glutamyl hydrolase (GGH) and folylpolyglutamate synthase (FPGS) are essential for maintaining intracellular folate homeostasis. Gamma-Glutamyl hydrolase (GGH), also

known as conjugase or folylpolyglutamyldihydrofolate hydrolase, is a gene that catalyzes the hydrolysis or the removal of folylpoly-gamma-glutamates and antifolylpoly-gamma-glutamates, including MTX polyglutamates. It gives 5-methyl-tetrahydrofolate to cleave gamma-polyglutamate chains that are attached to folate and antifolate (29). As shown in Fig 1.5, GGH conveys glutamyl groups, which allows folate to leave the cell.

Also important to maintaining folate homeostasis is folylpolyglutamate synthase (FPGS). FPGS is the gene that catalyzes the ATP-dependent addition of glutamate moieties to folate and converts them to polyglutamate derivatives. This enzyme has a crucial role in establishing and maintaining both cytosolic and mitochondrial folylpolyglutamate concentrations and, therefore, in the survival of proliferating cells. FPGS adds glutamyl groups to the folate molecule, which is essential for cellular retention of folate (29).



**F. Folate Deficiency:**

The importance of folate in growth and development, Folate deficiency is an important public health issue. In mouse studies, increased intake of folate has been associated with a decreased risk of many types of diseases. A review (Rosati et al.) evaluates the impact of folate depletion on blood and tissue folate status. They reported that the duration of feeding, dosage of folate, and use of antibiotics all impact the degree of folate depletion (30)

A great deal of research has been conducted to better understand the impact of folate deficiency on the body, but has tended to focus on folate concentration in serum. Folate is transported in the blood in the monoglutamate form. In tissues and cells, by contrast, folate is polyglutamated and deconjugation is required. The conjugase enzyme in tissues and cells is required in order to regulate the level of polyglutamation of folate within the cell. As such, folate deficiency in tissues and cells may result not simply from a lack of folate in diet, but also from issues related to the enzymes involved in conjugation and deconjugation of folate. Understanding folate deficiency therefore requires understanding the role of this process and enzymes in folate available in tissues and cells. Consequently, we must understand folate availability in the tissues and cells where these problems arise in order to effectively understand the roles of folate metabolism in health and disease. Most studies, which speculate on the link between folate deficiency and health, have evaluated serum folate status, without regard for tissue status. A comprehensive study needs to be conducted to investigate the impact of serum folate levels on tissue folate status. Our objective in this work is to begin evaluating this question.

## **CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS**

### **Purpose of Study:**

Several studies have measured tissue folate level in response to dietary depletion, and showed the effect of folate deficiency on tissue folate levels depending on the length and duration of the dietary intervention. A study by Watson and Margison et al., 2010 (31), showed that feeding rats a folate-deficient diet for a certain period of time caused moderate intracellular folate deficiency. Serum folate dropped by 40% and tissue folate by 25-60%. Liver folate was depleted by 30%, and colon folate by 60%. Our study should confirm these earlier results.

Further antibiotic use inhibits production of bacterially synthesized folate in the colon. By comparing the tissue folate status of mice treated with antibiotic versus those not treated with antibiotic, we can measure the effect of using the antibiotic, the contribution of bacterially-synthesized folate to folate tissue status, and the importance of using antibiotics in tissue folate status research.

The effect of folate status on the expression of genes is not adequately understood as it pertains to folate distribution within the cell. Studying the impact of folate status on the expression of genes responsible for folate retention in the cell will offer insight into the role of folate in a variety of DNA-related metabolic processes, and in turn, on the impact of folate deficiency on the development of health conditions.

This project seeks to understand the mechanism behind the effects of folate deficiency, and how cells and tissues regulate metabolic enzymes affected by folate when these cells and tissues are

under the stress of folate depletion. We hypothesize that the removal of folate from the diet will result in folate reduction in tissues. Our hypothesis has the following aims.

**Specific Aim 1:** We will determine whether folate deficiency has an impact on serum and tissue folate level in the absence of antibiotics.

**Specific Aim 2:** We will determine if the activity of succinyl sulfathiazole (antibiotic) affects the tissue folate status in response to folate depletion.

**Specific Aim 3:** We will determine whether folate status has an impact on the expression of genes responsible for folate retention in the cell.

### CHAPTER 3: MATERIALS AND METHODS

#### Animals:

C57B1/6 from Charles River, born in September 2013 and received in November 2013. They were put on chow diets up until started them on their respective diets in January 2014, at 4 months old.

**Table 3.1: Numbers of Animals and Types of Diets**

<b>Number of Animals</b>	<b>Weeks (w)</b>	<b>Type of Diet</b>	<b>Antibiotic (AB)</b>
3 animals	8 w	FA	No
3 animals	8 w	FA	Yes
3 animals	8 w	FD	No
3 animals	8 w	FD	Yes

**FA Diet:** AIN-93G purified rodent diet with vitamin free casein and 2 mg/kg Folic Acid; In addition, AIN-93G purified rodent diet with 1% succinyl sulfathiazole and 2 mg/kg Folic Acid.

**FD Diet:** Folate deficient AIN-93G purified rodent diet with vitamin free casein and 1% succinyl sulfathiazole. In addition, folate deficient AIN-93G purified rodent diet with vitamin free casein.

Animals were fed ad libitum, and diet was adjusted weekly as needed. Food intake was calculated weekly. The mice consumed on average 3-4g of diet per mouse per day. The animals

were sacrificed by CO<sub>2</sub> and cervical dislocation. Blood was collected and held at room temperature for 30 minutes to allow clotting, at which time serum was collected for folate detection by microbiological assay. All tissues (liver, kidney, spleen, colon, brain, small intestine, testis, and tail) were promptly removed and flash frozen in liquid nitrogen until used. Serum, colon, liver, kidney, brain, and small intestine were weighed and placed in 30 volumes of extraction solution: 0.7 B-Mercaptoethanol per one ml buffer 1 was prepared by dissolving 3.2 gram of sodium ascorbate into 19 ml dH<sub>2</sub>O and adding 1 ml potassium phosphate buffer (1mol/l, pH 6.1).

#### Tissue culture:

SV-40 transformed mouse embryonic fibroblasts (MEFs) Tag 92, BNL CL2 cells, which are transformed liver, cells derived from BALB/c mice (ATCC TIB-73<sup>TM</sup>) and Tag 207. Cells were maintained in either folate adequate or folate free media with dialyzed serum. In the beginning, cells grown in folate free media were cultured in media supplemented with 10uM thymidine/60uM adenosine (T/A) to allow cell survival. Removal of folate from the media without T/A supplementation initially resulted in cell death. Stepwise reductions in the percent T/A was conducted over time, such that T/A concentrations were decreased every 3 passages until T/A supplementation was not required for survival. Cells were counted using TC10 Automated Cell Counter, (Biorad, Hercules, CA) and were seeded at the identical densities.

#### **HARVESTING CELLS:**

The flasks of cells were first observed under the microscope to determine if the cells were 75% confluent. The old media from the cells was removed and the flasks were washed with 5 ml of pre-warmed 1X PBS-EDTA. Once the 1X PBS-EDTA was removed, 2 mL of the trypsin was added. The flasks were then incubated for approximately 2-3 minutes at 37° C in order to detach the cells from the bottom of the flasks. The cells were again viewed under the microscope to ensure that the cells were lifted off the flask. To stop the trypsin from working, we added 3 mL of complete growth media. By using a serological pipette, the cells were pipetted up and down several times to separate the cells from each other. The cells, along with the added complete media, were transferred into a 15 mL conical tube for washing. The cells were centrifuged for 5 minutes at 1300 rpm at 4° C. The media was carefully removed without disturbing the white pellet visible on the bottom of the conical tube, and 5 mL of 1X PBS was added. The cells were again centrifuged at 1300 rpm for 5 minutes at 4° C and the 1X PBS was carefully removed without removing the pellet.

**RNA Isolation:**

After the cell was harvested, we isolated the whole RNA by using Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD). First, we allowed the samples either from cell or tissue culture to incubate in Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD) until the next day, under 4° C. The next day, we spun the samples with the Isol-RNA lysis at 12000g for 10 minutes at 4° C. At this point, there were three layers in each tube: the top layer is clear and is called the aqueous layer; the middle layer, which contains the precipitated DNA, is white and is called the interphase; and the bottom layer, containing organic material, is the pink organic layer. The DNA pellet went down the tube and we collected the supernatant, and we carefully pipetted off the aqueous phase with the larger pipette, being careful to not draw any of the organic or DNA phase. Then we added 2000 µL of chloroform for every 1 mL of Isol-RNA Lysis Reagent to each sample (supernatant), shook it

vigorously for 20 seconds, and incubated samples at room temperature for 2-3 minutes. After incubation, we centrifuge all samples at maximal speed (12000 x g) for ten minutes at 4° C.

We then transferred the aqueous phase to a fresh tube. The aqueous phase is the colorless upper phase that corresponds to 60% of the volume of Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD) used. Then we added 0.5 mL of isopropanol to the aqueous phase and mixed it gently. We then let the samples sit at room temperature for 10 minutes and centrifuged them for 10 minutes at (12,000 x g) at 4° C. After extracting the tubes from the centrifuge, we removed the supernatant from each sample, and placed the tubes on ice. There was a pellet barely visible at the bottom of each tube. We poured off the isopropanol and added 1 mL of 75% EthOH in DEPC treated water and mixed samples by flicking and inverting the tubes or by vortexing. The pellet came loose from the bottom of the tube, but remained intact. Then we centrifuged at 7500 Xg for 5 minutes at 4° C. After that we needed to remove all the ethanol from each tube, but not disrupting the pellet. At the end, we added 30 µL of RNase free water to re-dissolve the pellet. If the pellet was not dissolved, we tried to pipette gently up and down, then we incubated the tubes at 4° C for same day use or at -80° C for second day use. Finally we quantified our samples of RNA by using the Nano drop to check the concentration of the RNA, and then we were ready to the next step, which was CDNA synthesis.

## CDNA SYNTHESIS:

After the isolation of whole RNA from cells, we synthesized cDNA from the RNA we isolated. First, we needed to prepare the reverse transcription reaction mix by combining the following components of the RT-PCR kit (Perkin Elmer, Waltham, MA) in a sterile 1.5 ml microcentrifuge tube on ice:

Master Mix:

Master Mix: transcription reaction mix by combining	5uL
Reverse Transcriptase (RT) (Perkin Elmer, Waltham, MA)	4uL
Reverse Transcriptase (RT) (Perkin Elmer, Waltham, MA)	4uL
dNTP (Perkin Elmer, Waltham, MA)	1uL
Reverse Transcriptase (RT) (Perkin Elmer, Waltham, MA)	1uL

We prepared enough mix to allow 15 uL for each cDNA synthesis reaction to be performed, and vortexed them gently, keeping samples on ice prior to dispensing them into the reaction tubes. Then, we aliquoted 15 uL of the reverse transcription reaction mix to each reaction tube on ice accordingly. During the experiment, we used a specific amount of the RNA. We combined the RNA (1000 ng) and the cDNA random primer in nuclease-free water for a final volume of 5 uL per RT reaction.



1. Reaction Primer= 1 $\mu$ L
2. RNA = 1-3 (depending on concentration)
3. DH<sub>2</sub>O = certain volume to make total equal to 5  $\mu$ L
4. Total = 5

Then we placed the tube into a preheated heat block at 70° C for 5 minutes. After that we removed the tubes and put them on ice immediately for 5 minutes, and then we spun them for 10 seconds to collect the condensate. Then we added the RNA and mix to the reaction tubes containing RTase reaction mix, then we centrifuged for 1 min to mix the samples. Next, we placed the samples in the thermocycler (Eppendorf North America, NY) and set parameters. Certain temperatures were assigned at each step. Finally, we purified the cDNA samples with QIA quick PCR purification kit (Qiagen, Valencia, CA) and quantified the cDNA using NANO-DROP (Eppendorf North America, NY).

**REAL TIME PCR:**

Each PCR reaction consisted of 2  $\mu$ L of purified cDNA, 4mM MgCl<sub>2</sub>, 0.5 $\mu$ M each of sense and antisense primers, and 2  $\mu$ L of FastStart DNA master SYBR Green I enzyme-SYBR reaction mix (Roche). The parameters for all of the amplifications are detailed in Table 2. All the transcripts were normalized to the housekeeping gene, RPL-4, and GAPDH.

Table 3.2: Real time PCR parameters for all amplification

	Duration	Temperature
Initial denaturing	10 min	99° C
Denaturing	10 sec	96° C
Annealing	10 sec	62° C
Extension	5 sec	72° C
Melting		40° C-99° C

## Folate Microbiological Assay and Overview of the *Lactobacillus Casei*

After the treatment of cells and tissues with the methods described above we used the microbiological assay to determine the intracellular folate level. The level of folate was measured using the *Lactobacillus casei* (ATCC 7469) microbiological assay in 96 well microtiter plates in which results were read with an automatic plate reader, as described by Home et al (Horne and Patterson 1988). The growth of the *Lactobacillus casei* (L.casei) bacteria depends on the folate concentration. The growth of bacteria increases, as there is more folate in the growth media. We used the growth curve of the bacteria to measure the folate concentration in our samples.

The folinic acid (calcium salt) [(6-ambo)-5-HCO-H<sub>4</sub>PteGlu] was prepared in water at a concentration of 6 mM ( $6 \times 10^6$  fmol/ $\mu$ l) and first diluted to  $6 \times 10^3$  fmol/ $\mu$ l, then diluted to 60 fmol/ $\mu$ l, and at the end to a final dilution of 2 fmol/ $\mu$ l (working solution). The single strength folic acid casei medium was prepared by dissolving 9.4 gram of folic acid casei medium powder and 50 mg of sodium ascorbate (Vitamin C) in 100 mL of diH<sub>2</sub>O. The mixture was then filtered through a 0.22- $\mu$ m sterile syringe filter. The working buffer was prepared by dissolving 3.2 gram of sodium ascorbate in 19 ml diH<sub>2</sub>O and by adding 1 mL potassium phosphate buffer (1mol/l, pH 6.1). The solution was then filtered through a 0.22- $\mu$ m sterile syringe filter. Finally, the L.casei inoculum was cultured in a growth medium supplemented with 0.025% sodium ascorbate and 0.6 $\mu$ g/l folinic acid calcium salt.

One mL of L.casei was diluted in 5 mL of growth medium to incubate overnight at 37°C. The next day, the OD was measured and diluted to an OD value of 0.5 (standard OD value of the L.casei inoculum). The 96-microtiter plates was then set up as shown in table 3.3 and 3.4. Lights were turned off due to folate light sensitivity. Two fmol/ $\mu$ l of folinic acid calcium salt as standard was added (0,10,20,40,60,80,100,120 fmol in each well) to each designated well, a diluted sample was added to

each designated well, and at the end, 20µl of L.casei was added to all wells except the blank ones. The light sensitive samples were wrapped with parafilm in order to keep the humidity within the microtiter plate. The plate was also covered with aluminum foil to prevent the degradation of folate due to light.

The plate was incubated overnight at 37°C for approximately 18 hours. The plate was read at an absorbance of approximately 570-635 using TECAN-GENios plus plate reader and TECAN Magellan software. Folate concentration was calculated by comparing with the standard. Folate concentration was considered significantly different from control at a p-value < 0.05.

### Table 3.4 Folate Assay Microtiter Plate Setup

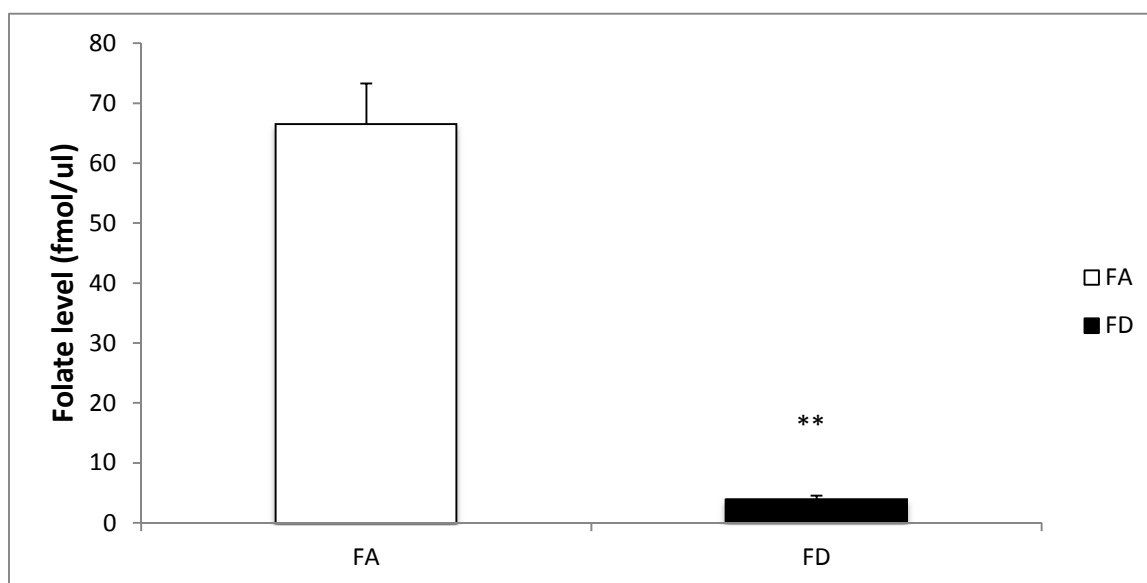
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**Statistical Analysis:**

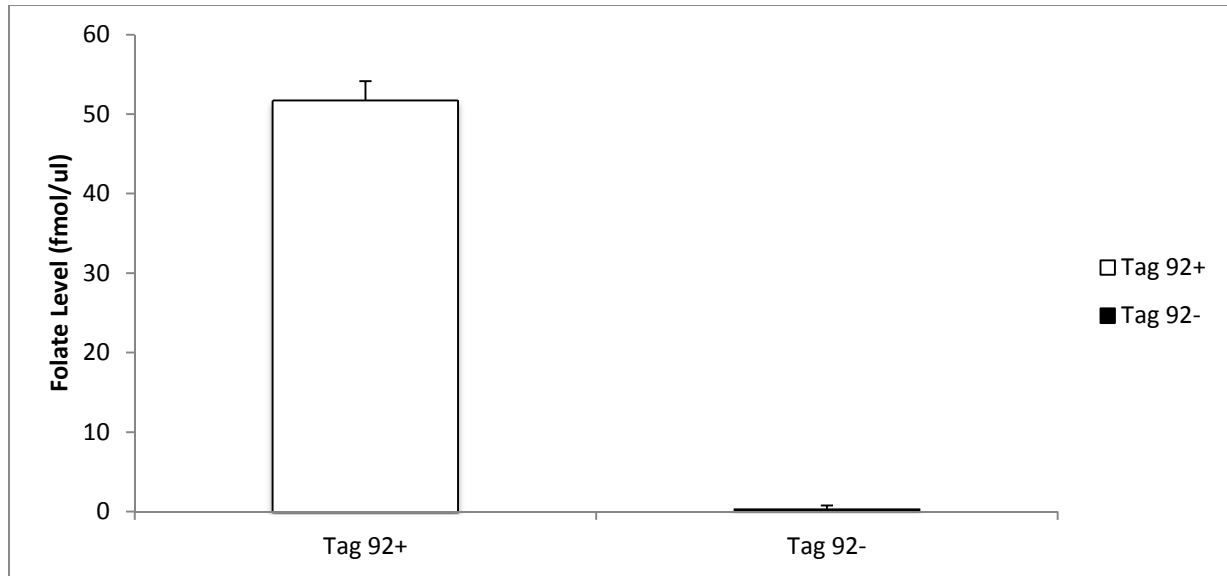
Statistical significance between means was determined using t-test. P values less than 0.05 were considered statistically significant.

## CHAPTER 4

## RESULTS

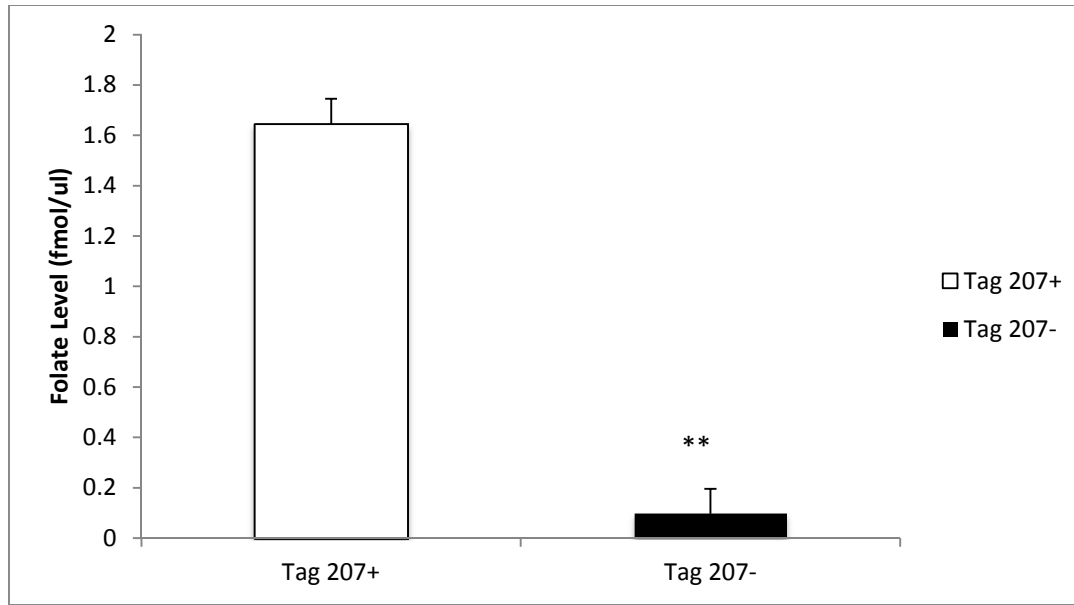


**Figure 5: Folate level in BNL2 in response to folate depletion.** The cells were grown in the presence of folate (FA) while a second set were grown in folate depleted media (FD). Folate level was determined by L.Casei microbiological assay. Data were triplicated and was presented as mean  $\pm$  S.E.M. Statistical significant was determined by T-Test. \*\*  $p < 0.001$

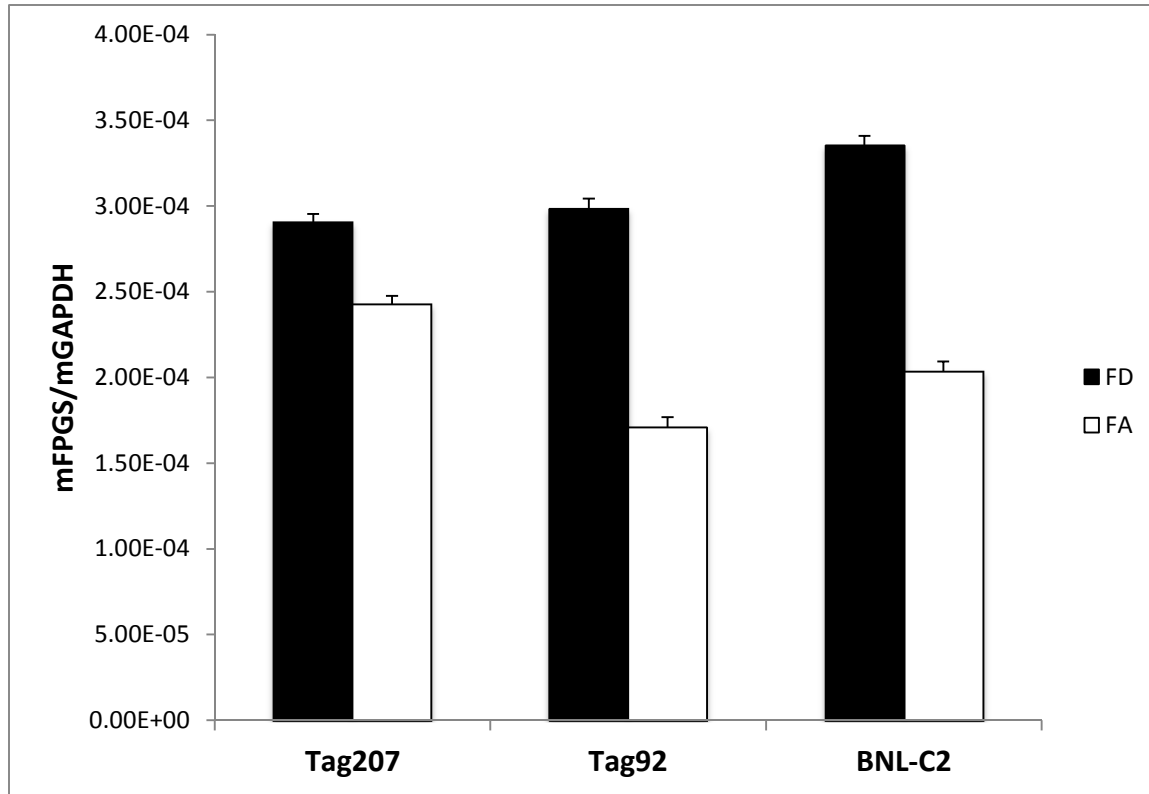


**Figure 6: Folate level in UNG +/+ and UNG -/- MEFs in response to folate depletion.** The cells were grown in presence and absence of folic acid media. Folate level was determined by L.Casei microbiological assay as described in methods. Data were triplicated and was presented as mean  $\pm$  S.E. M. Statistical significant was determined by T-Test. \*\* P value  $< 0.001$ .

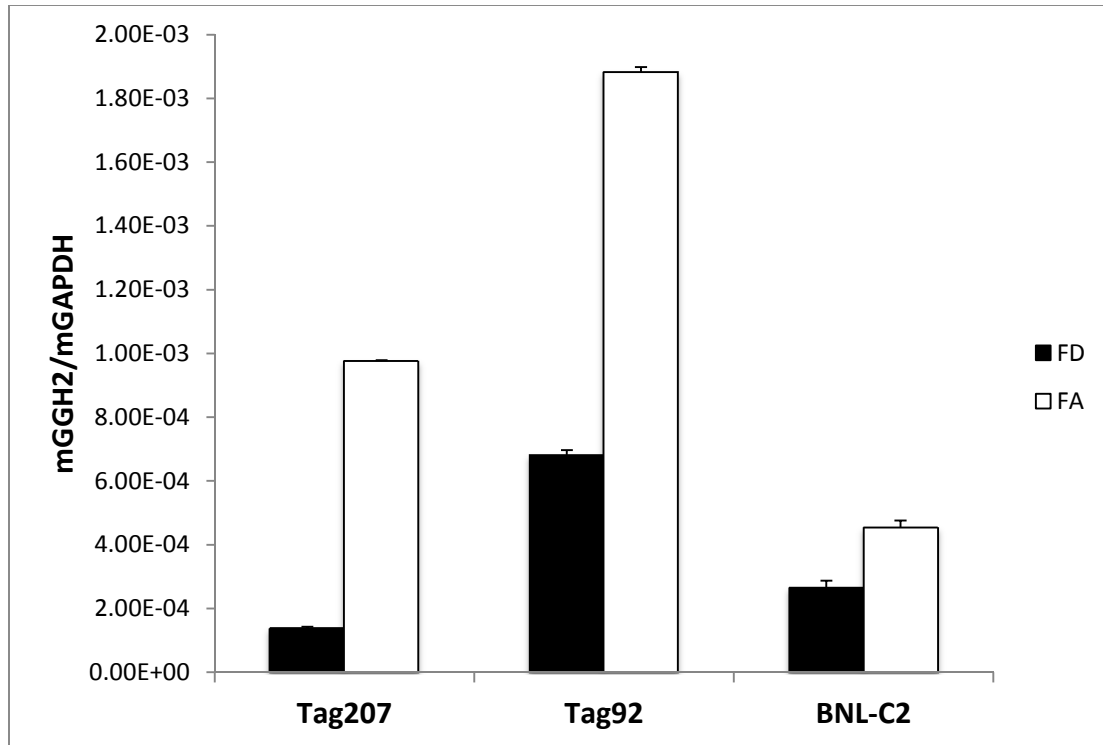




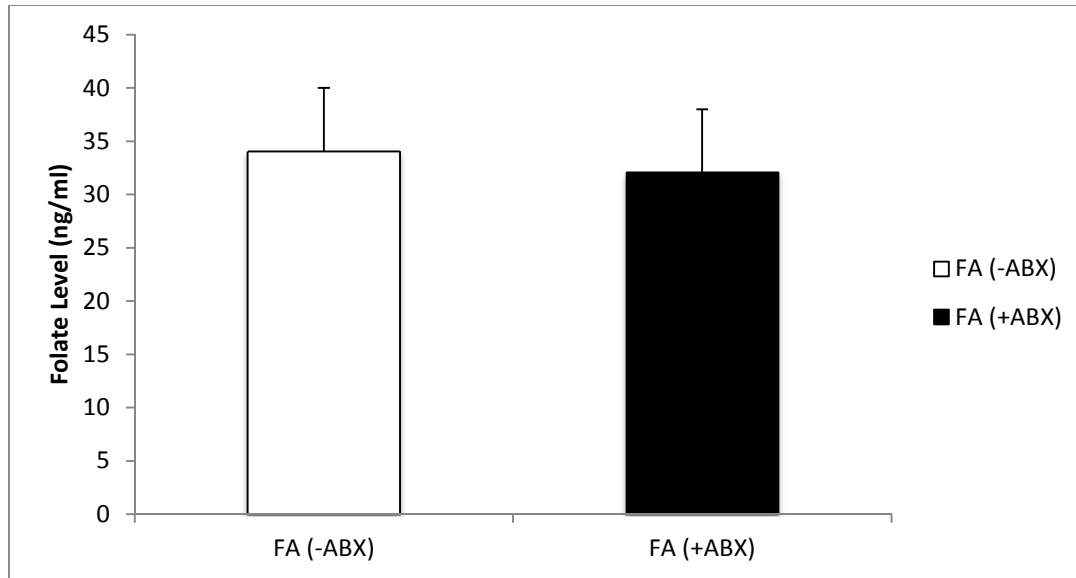
**Figure 7: Folate Level in Ung  $-/-$  MEFs in response to folate depletion.** The cells were grown in the presence and absence of folic acid media. Folate level was determined by L.Casei Microbiological assay as described in methods. Data were triplicated and was presented as mean  $\pm$  SEM. Statistical significance was determined by T-Test. \*\*P value  $<0.001$ .



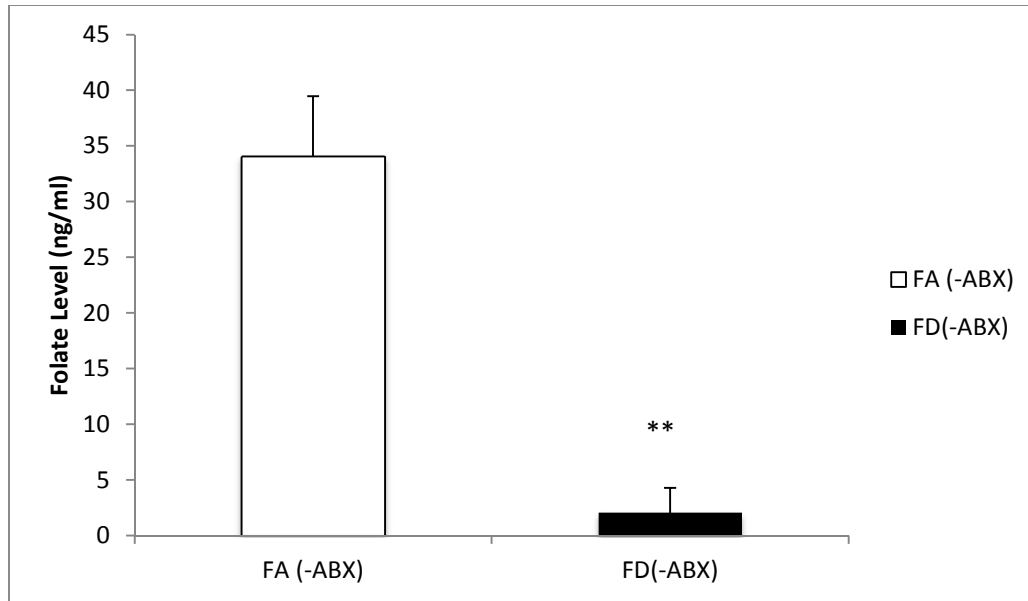
**Figure 8: Relative mFPGS gene expression in response to Folate depletion in UNG  $-/-$  MEF, Ung  $+/+$  MEF, and BNL-C2 cells.** Cells were grown in presence and absence of Folic acid in media. RNAs were isolated from cells grown for 3 passages. mGGH expression were determined by RT-PCR. Data were normalized to mGAPDH. Data were triplicated and presented as mean  $\pm$  SEM. Statistical significant was determined by T-test. \*\*  $P < 0.001$ . FPGS was higher in Folate depleted cells (0.84 fold higher in Tag 207 cells, 0.57 fold higher in Tag 92 cells, and 0.61 fold higher in BNL-



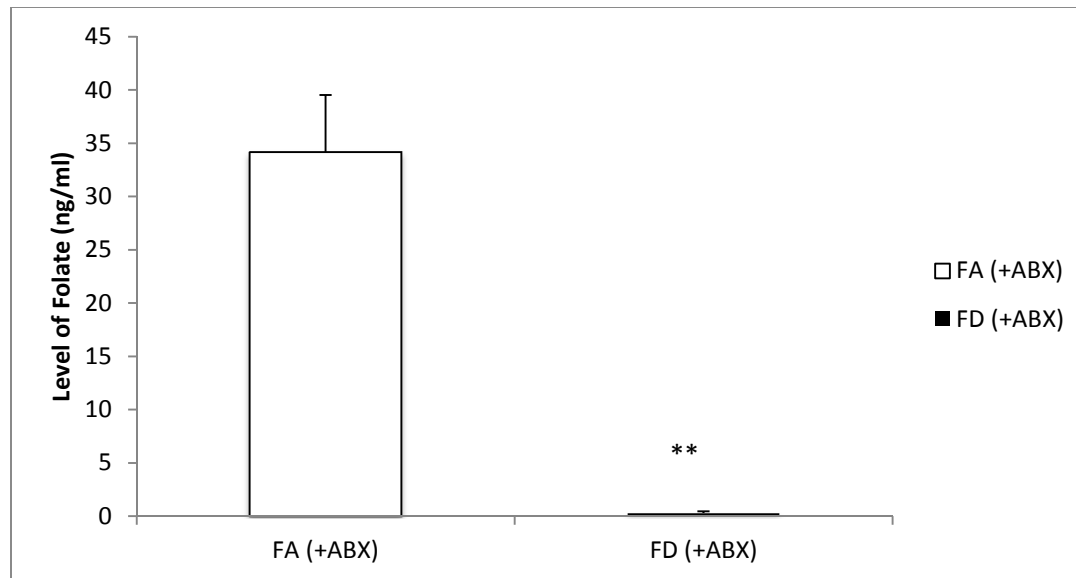
**Figure 9: Relative mGGH gene expression in response to Folate depletion in Ung  $-/-$  MEF, Ung  $+/+$  MEF, and BNL-C2 cells.** Cells were grown in presence and absence of Folic acid in media. RNAs were isolated from cells grown for 3 passages. mGGH expression was determined by RT-PCR. Data were normalized to mGAPDH. Data were triplicated and presented as mean  $\pm$  SEM. Statistical significant was determined by T-test. \*\*  $P < 0.001$ . mGGH was lower in Folate depleted cells ( 7 fold lower in Tag 207 cells, 2.7 fold lower in Tag 92 cells, and 1.7 fold lower in BNL-C2).



**Figure 10: Effect of Antibiotics on Serum Folate Level.** A comparison between group of mice fed folate- adequate diets without using antibiotics and group of mice fed folate- adequate diet treated with antibiotics. Folate levels in both groups were measured using the microbiological assay as described in methods. The data are expressed as the means  $\pm$  S.E. for data obtained from control groups. Statistical Significance was determined by T-Test.

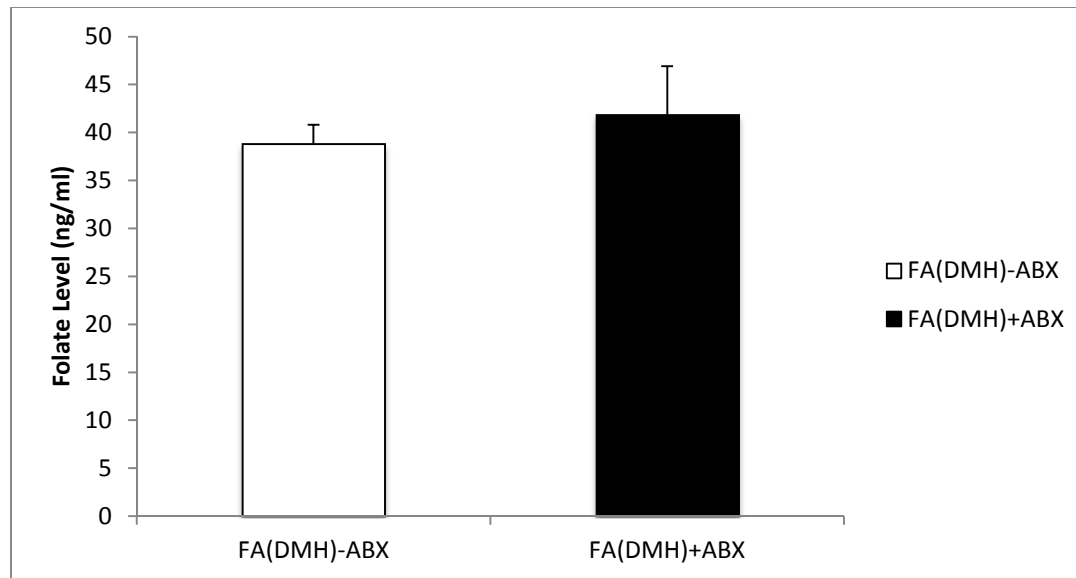


**Figure 11: Effect of Folate Depletion without antibiotics on serum folate level.** A comparison between FA (-ABX) mice group, containing folate adequate diet without using antibiotics and FAD (+ABX) mice group containing folate depleted diet without antibiotics. Folate levels in both groups were measured using the microbiological assay as described in methods. The data are expressed as the means  $\pm$  S.E. for data obtained from control group. Statistical significance was determined by T-Test. P value  $<0.005$ .

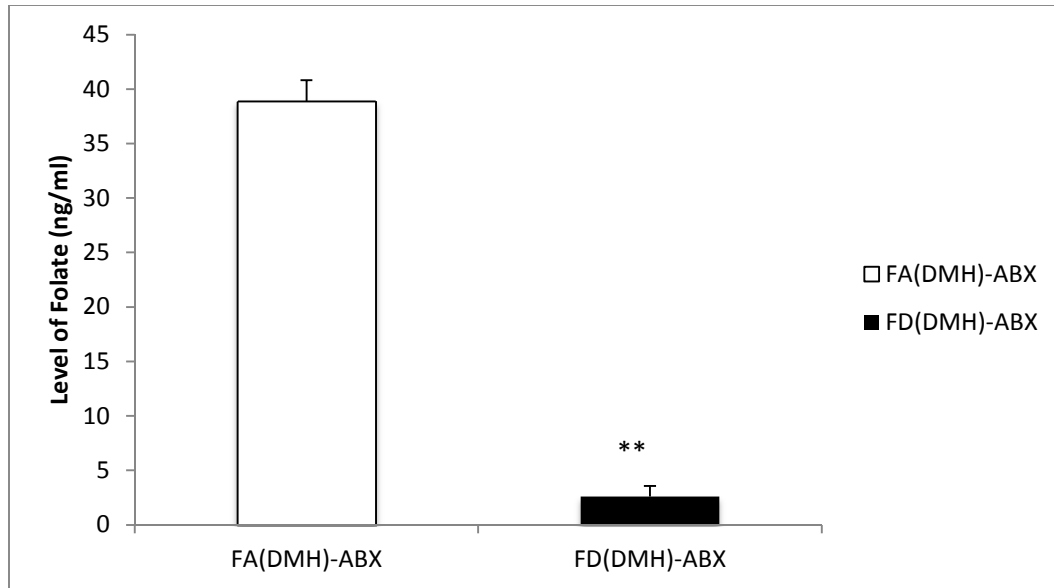


**Figure 12: Effect of folate depletion with antibiotics on serum folate level.**

Animal groups: mice group was implemented with folate -adequate diet with antibiotics and mice group was implemented with folate- deficient diet. Folate level was measured using the microbiological assay as described in methods. The level of folate is very low (near 0) when the animals were fed folate-deficient diet with antibiotics compared to mice group fed folate-added diet with antibiotics. The data are expressed as the means  $\pm$  S.E. For data obtained from 10 mice in each group. Statistical significance was determined by T-Test. P value  $< 0.001$ .

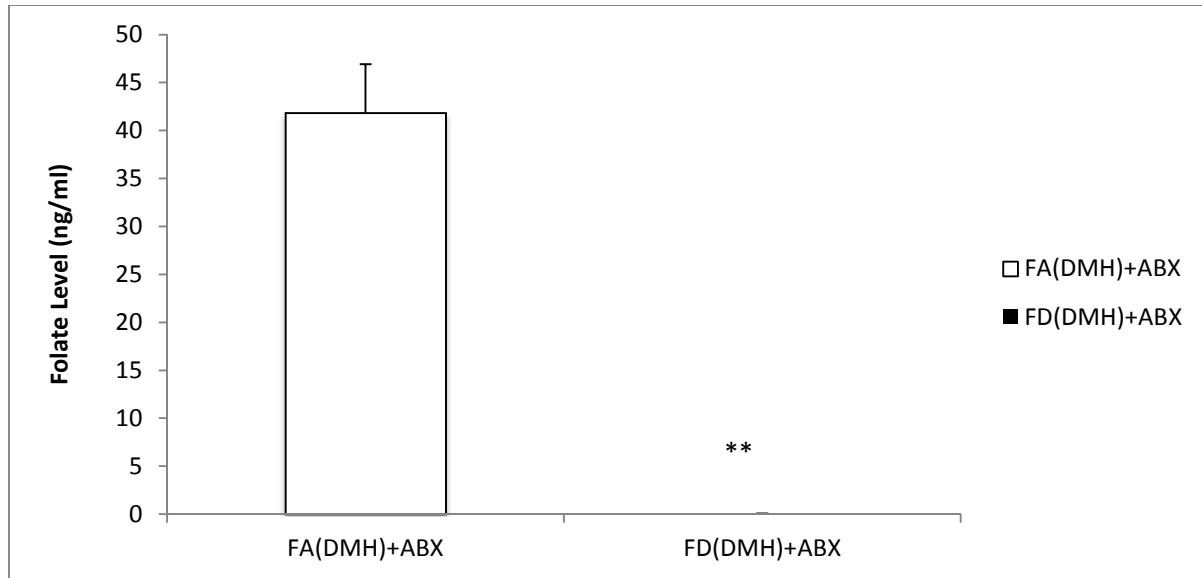


**Figure 13: Effect of DMH on serum Folate levels in folate added animals with and without antibiotics.** Experimental design: mice group were fed folate-adequate diet (2mg/kg, FA) for eight weeks. Mice were injected with DMH for 8 weeks. FA (DMH)-ABX group, were subjected to non-Antibiotics treatment and FA (DMH)+ABX group were subjected to a treatment with antibiotics. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from DMH treated groups. Statistical significance was determined by T-Test.

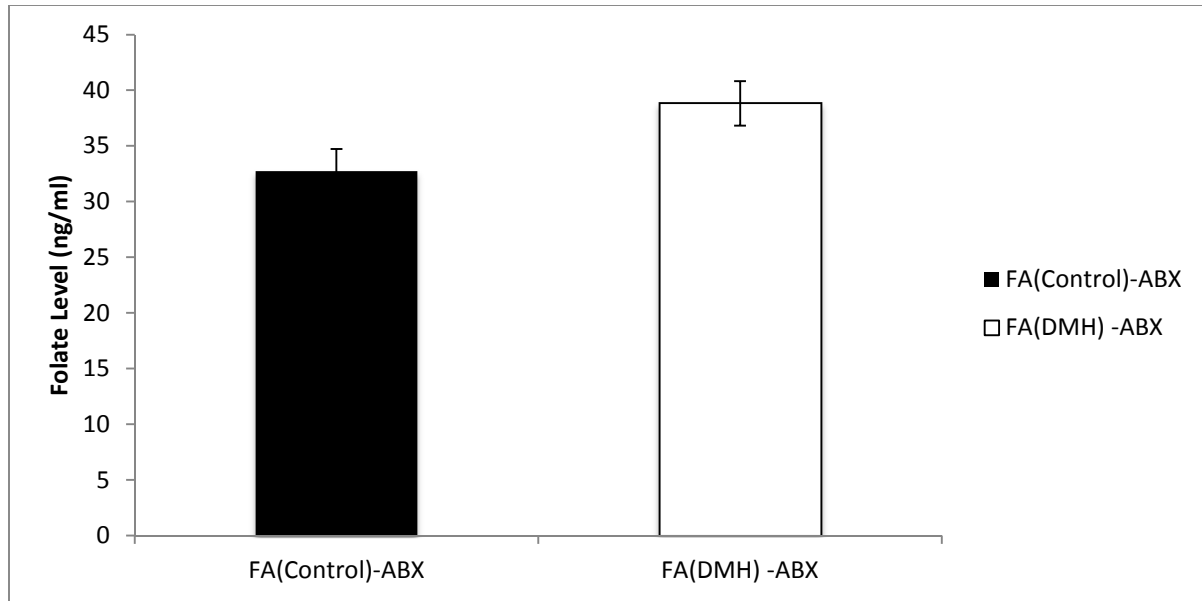


**Figure 14: Effect of DMH on serum folate levels in folate added and folate depleted animals without antibiotics.** Experimental design: mice were fed either a folate-adequate (2 mg/kg, FA) or folate-deficient (0 mg/kg, FD) diet for 8 weeks. After one week of ingestion of the respective diets, mice were injected with DMH. FA (DMH)-ABX group, were subjected to DMH treatment without antibiotics, mice group FD (DMH)-ABX were subjected to DMH treatment without antibiotics. Folate level was determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from groups treated with DMH. Statistical significance was determined by T-Test. P value < 0.001.

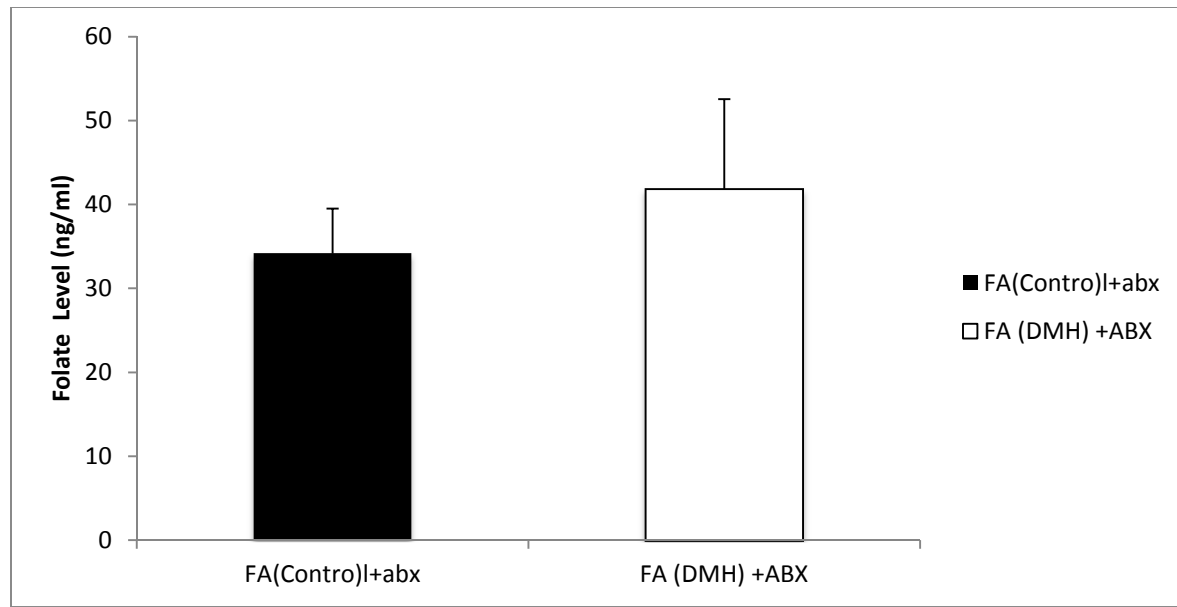




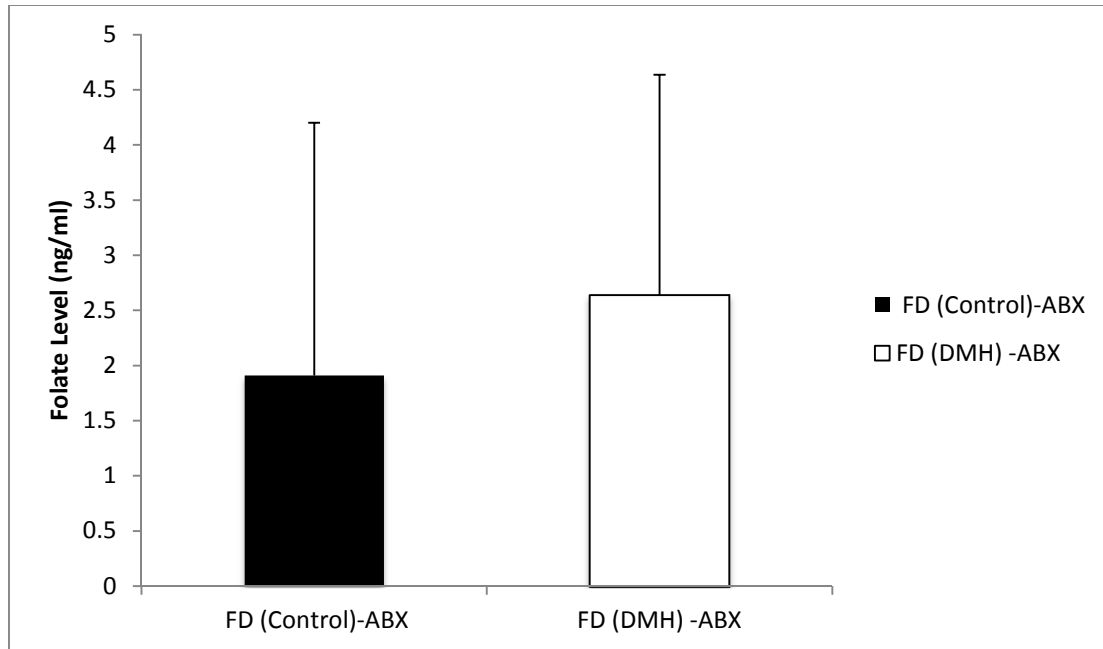
**Figure 15: Effect of DMH on serum folate levels in folate added and folate depleted animal groups with Antibiotics.** Experimental groups were fed either folate-adequate (2 mg/kg, FA) or a folate-deficient diet (0 mg/kg, FD) diet for 8 weeks. After one week of ingestion of the respective diets, mice were injected with DMH. Animal group were subjected to DMH treatment with antibiotics, and another animal group were subjected to DMH treatment with 1% succinyl sulfathiazole. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from groups treated with DMH. Statistical significance was determined by T-Test. P value  $< 0.001$ .



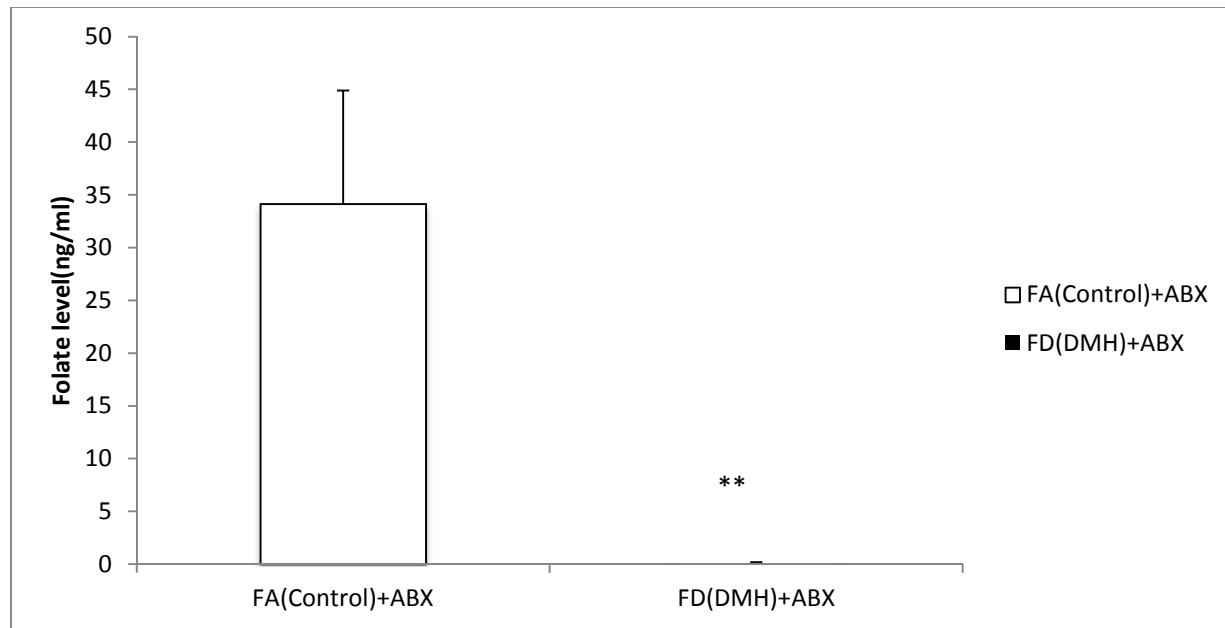
**Figure 16: Effect of DMH on folate serum level without antibiotics.** Experimental design mice group mice were fed folate-adequate (2 mg/kg, FA) diet. Animals were subjected to either no treatment (control) or treatment with DMH but not subjected to 1% succinyl sulfathiazole. After one week of ingestion of the respective diets, mice were injected with DMH. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from groups treated with DMH. Statistical significance was determined by T-Test. P value  $< 0.5$ .



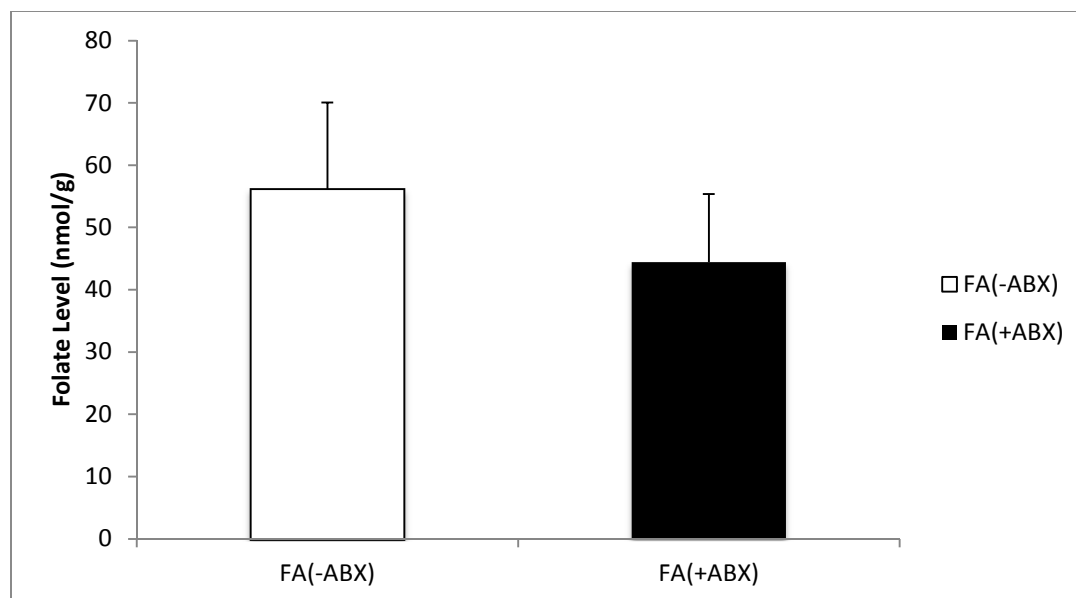
**Figure 17: Effect of DMH on folate serum level with antibiotics.** Experimental design animal group mice were fed folate-adequate (2 mg/kg, FA) diet. Animals were subjected to either no treatment (control) or treatment with DMH subjected to 1% succinyl sulfathiazole. After one week of ingestion of the respective diets, mice were injected with DMH. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from control and DMH treatment groups. Statistical significance was determined by T-Test.



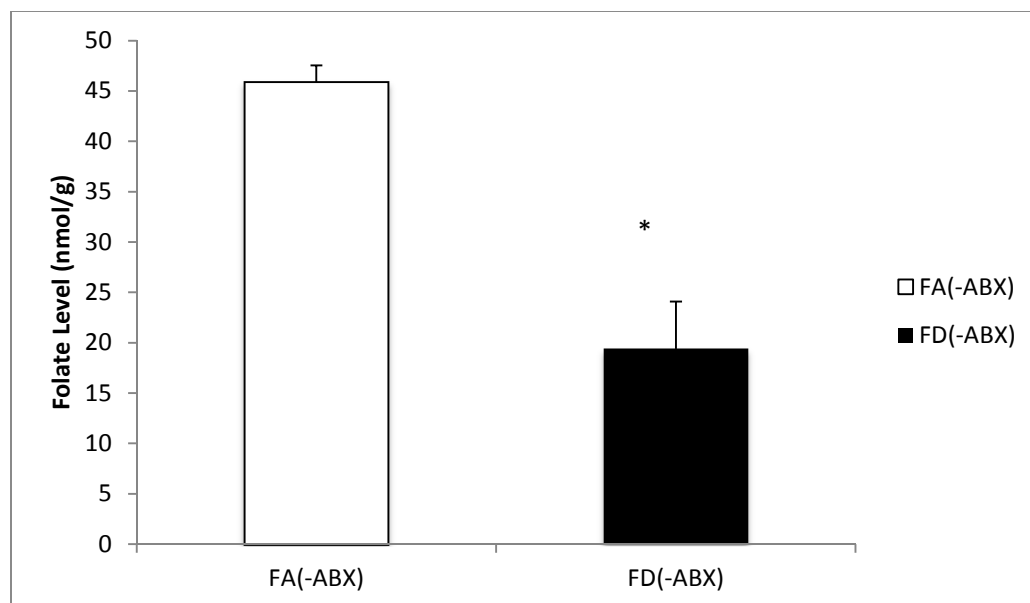
**Figure 18: Effect of DMH on folate serum level without antibiotics.** Experimental design animal group mice were fed folate-deficient (0 mg/kg, FD) diet. Animals were subjected to either no treatment (control) or treatment with DMH not subjected to 1% succinyl sulfathiazole. After one week of ingestion of the respective diets, mice were injected with DMH. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from control groups and groups treated with DMH. Statistical significance was determined by T-Test.



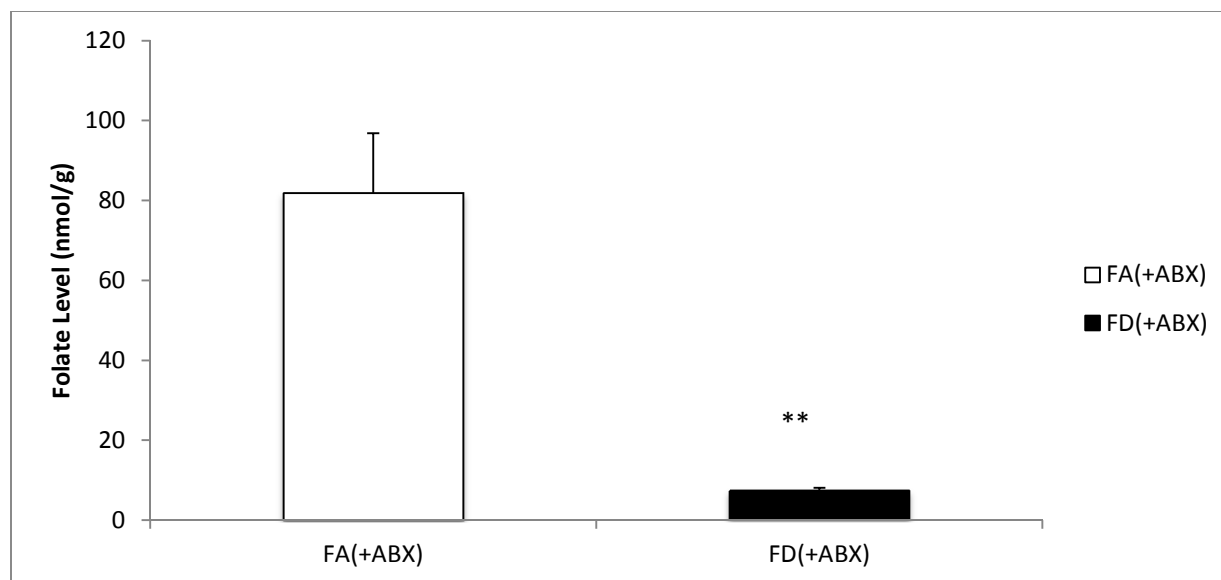
**Figure 19: Effect of DMH on folate serum level with antibiotics.** Experimental design animal group were fed folate-deficient (0 mg/kg, FD) diet. Animals were subjected to either no treatment (control) or treatment with DMH subjected to 1% succinyl sulfathiazole. After one week of ingestion of the respective diets, mice were injected with DMH. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from groups treated with DMH. Statistical significance was determined by T-Test. P value  $<0.001$ .



**Figure 20: Impact of Antibiotics on folate status liver tissue.** Liver folate distribution in mice fed folate-adequate diet without antibiotics and in mice fed folate-adequate diet treated with antibiotics. Folate level was measured using the microbiological assay as described in methods. The data are expressed as the means  $\pm$  S.E. For data obtained from DMH group. Statistical significance was determined by T-Test. P value  $< 0.5$ .

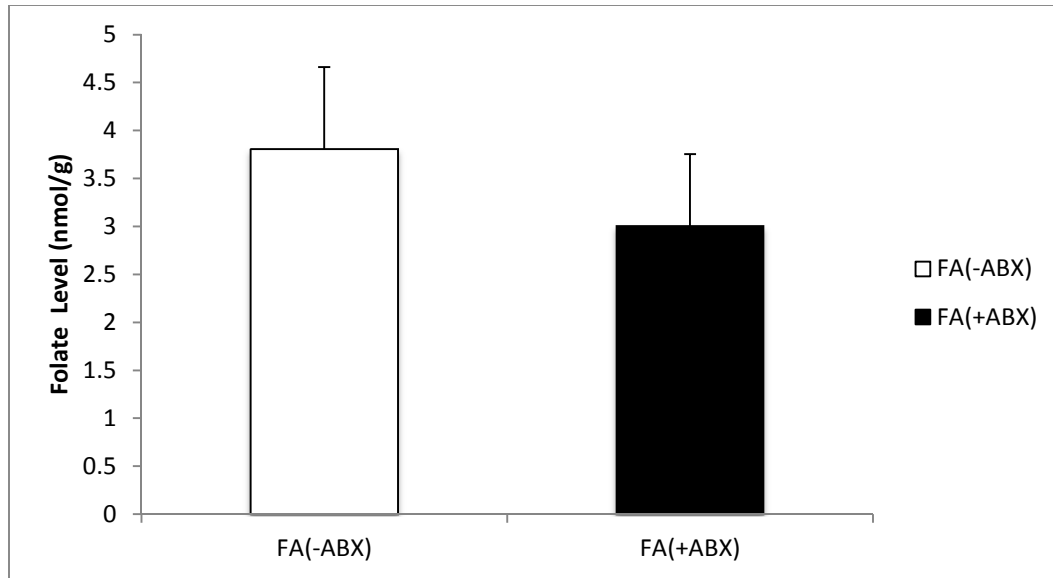


**Figure 21: Effect of Folate Depletion without Antibiotics on liver tissue sample.** Liver folate distribution in mice fed folate-deficient diet without using antibiotics compared to a group of mice fed folate-adequate diet without using antibiotics. Folate level was measured using the microbiological assay as described in methods. The data are expressed as the means  $\pm$  S.E. For Data obtained from control 1 group. Statistical significance was determined by T-Test. P value  $<0.005$ .

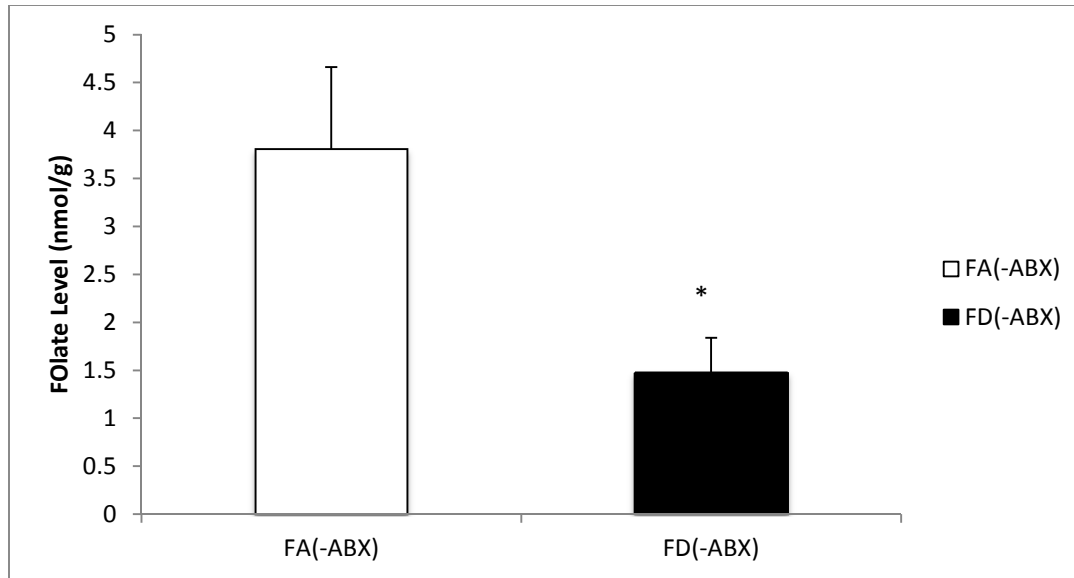


**Figure 22: Effect of Folate Depletion With Antibiotics on liver tissue sample.** Liver tissue was treated with antibiotics showing the relative folate depletion. Control group fed folate-adequate diet treated with antibiotics and control group fed folate-deficient diet treated with antibiotics. Folate level was measured using the microbiological assay as described in methods. The data are expressed as the means  $\pm$  S.E. For data obtained from control group. Statistical significance was determined by T-Test. P value <0.005.

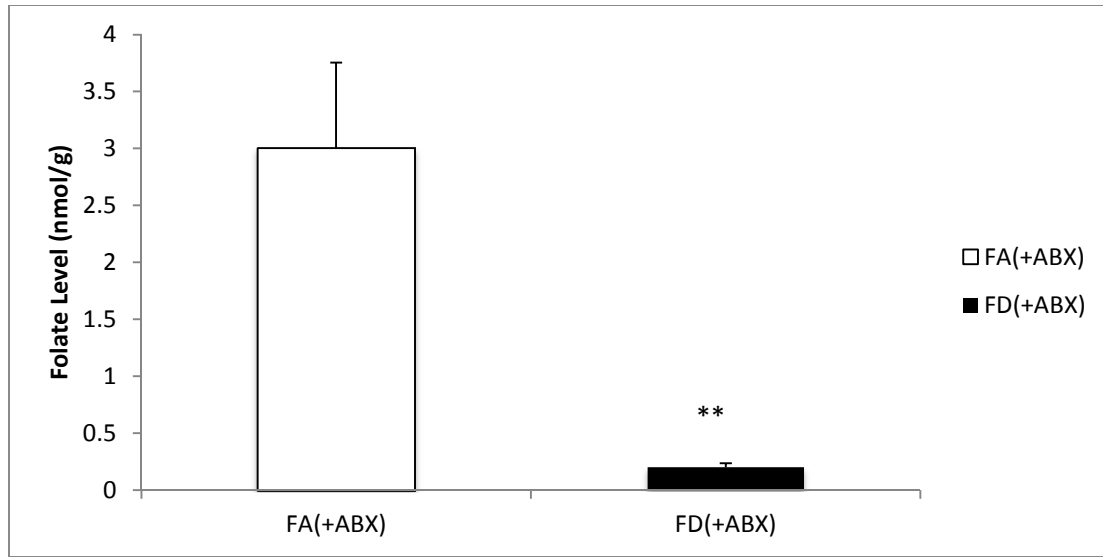




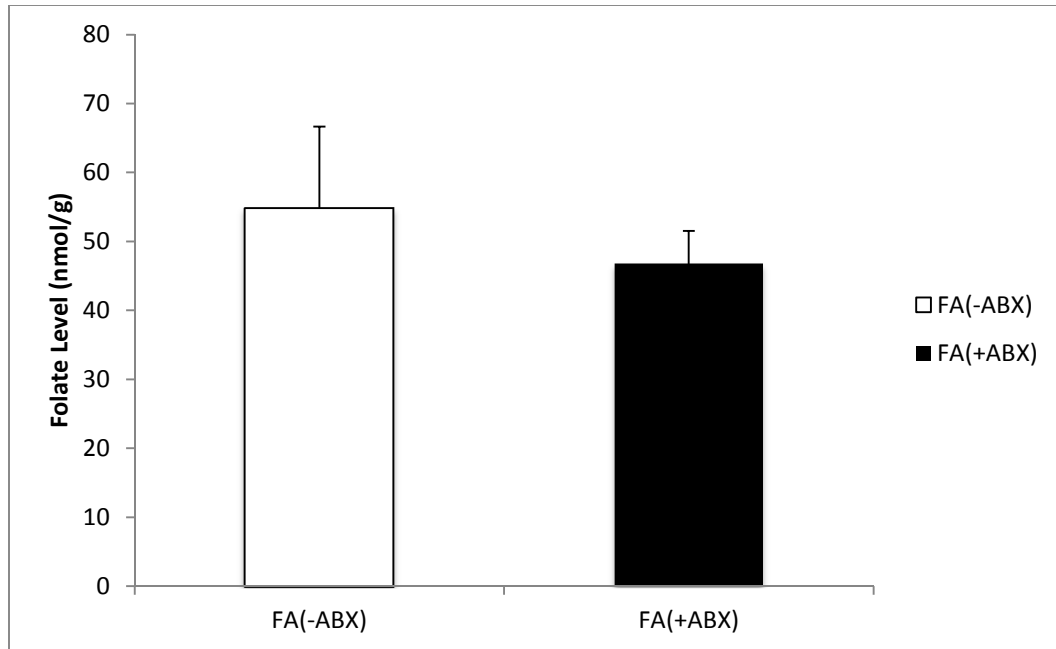
**Figure 23: Effect of Antibiotics on Colon Folate Distribution.** Colon tissue was treated with antibiotics showing the distribution of folate adequate diet. Control group fed folate-adequate diet without treatment of antibiotics compared to control group fed folate-adequate diet treated with antibiotics. Folate level was measured using the microbiological assay as described in methods. The data are expressed as the means  $\pm$  S.E. For data obtained from control group. Statistical significance was determined by T-Test.



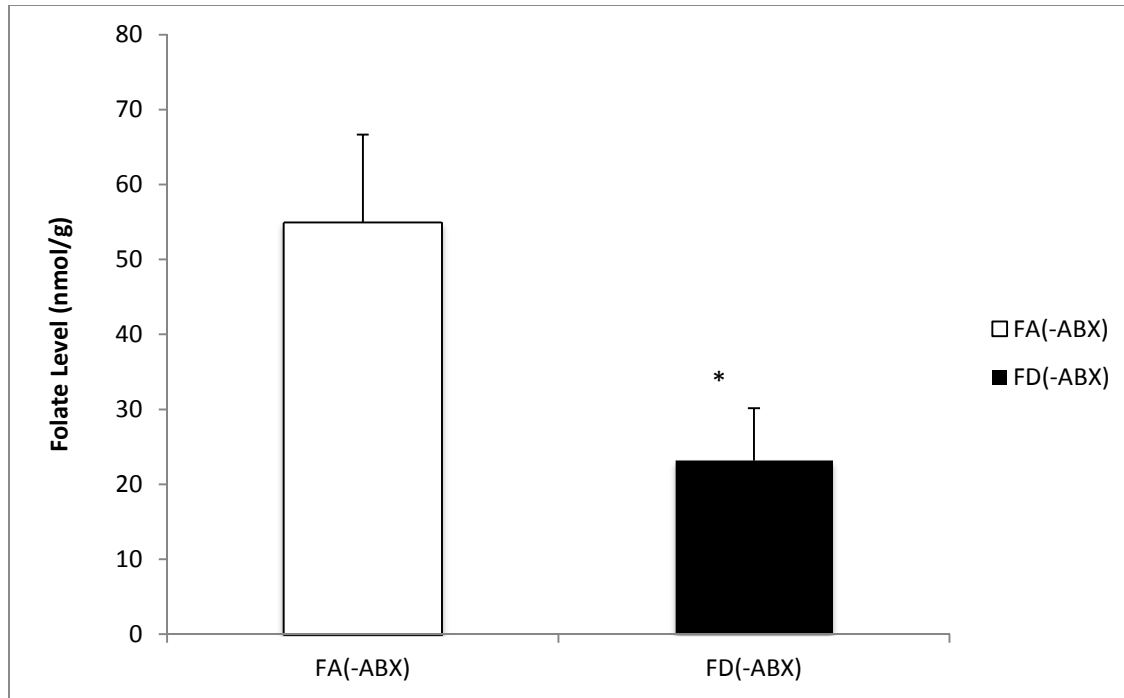
**Figure 24: Effect of Folate depletion Without Antibiotics on colon tissue sample.** Control group fed folate-adequate diet without antibiotics compared to a control group fed folate-deficient diet without antibiotics. The relative level of folate was determined using the microbiological assay as described in methods. The data are expressed as the means  $\pm$  S.E. For data obtained from control group. Statistical significance was determined by T-Test. P value  $< 0.05$ .



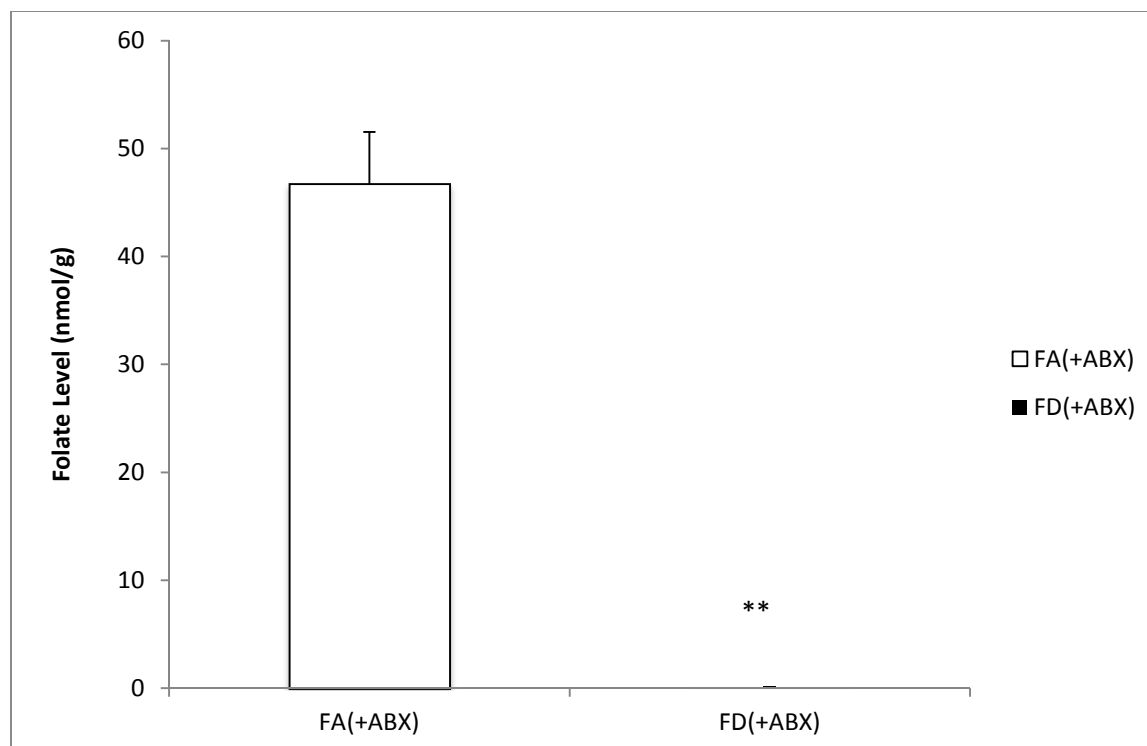
**Figure 25: Effect of Folate Depletion with Antibiotics on colon tissue sample.** Mice were treated with antibiotics. A comparison between control group fed folate-adequate diet treated with antibiotics and control group fed folate deficient diet treated with antibiotics. Folate level was measured using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from control group. Statistical significance was determined by T-Test. P value  $<0.05$ .



**Figure 26: Effect of Antibiotics on Kidney Folate Distribution.** A comparison between group fed folate-adequate diet without using antibiotics and group fed folate-adequate diet treated with antibiotics. Folate level was determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from DMH treated groups. Statistical significance was determined by T-Test.

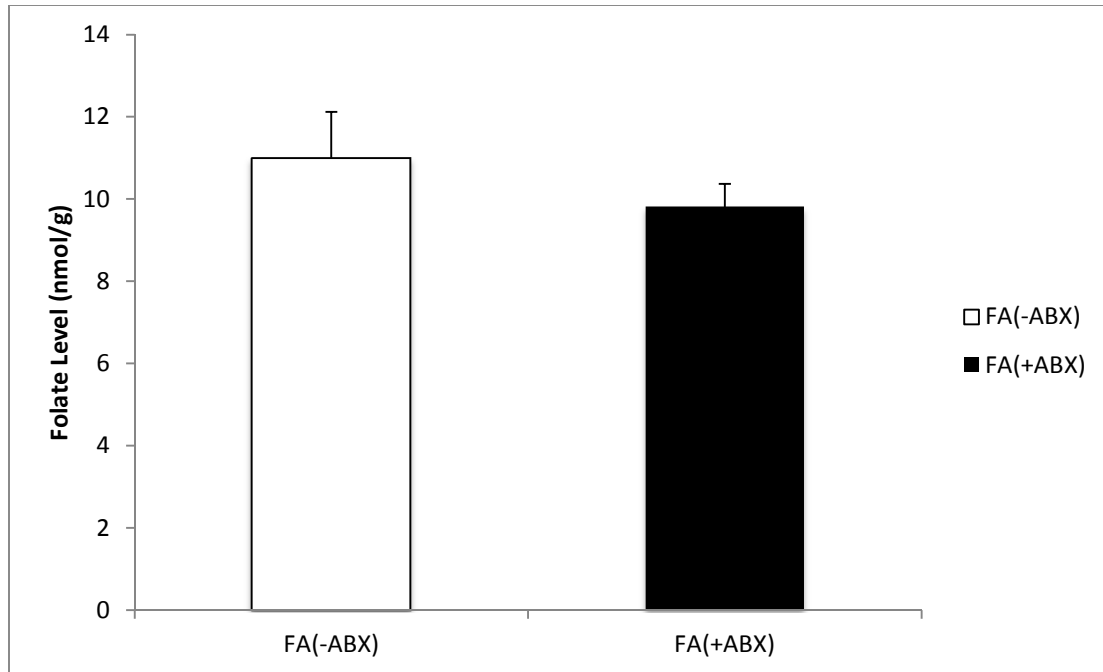


**Figure 27: Effect of Folate Depletion Without Using Antibiotics on kidney tissue sample.** Two groups of mice: group fed folate-adequate diet without using antibiotics and group fed folate-deficient diet treated without antibiotics. The relative level of folate in kidney tissue was measured using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from DMH group. Statistical significance was determined by T-Test. P value  $<0.05$

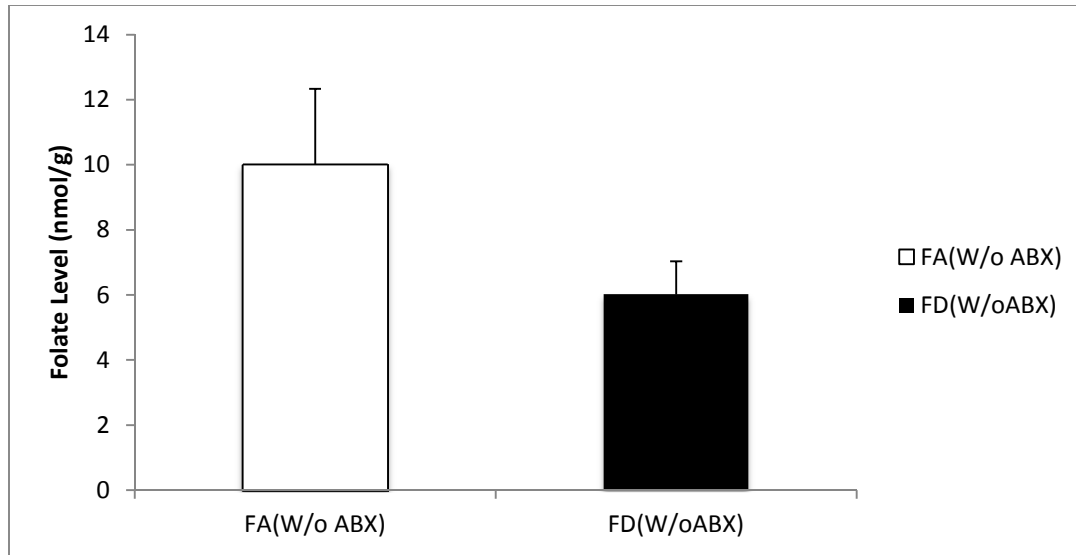


**Figure 28: Effect of Folate Depletion with Antibiotics on Kidney Tissue sample.**

The impact of antibiotics on group fed folate-adequate diet treated with antibiotics and group fed folate-deficient diet treated with antibiotics. The relative level of folate in kidney tissue was measured using the microbiological assay as described in methods. Values represent a mean  $\pm$  S.E. for data obtained from DMH group. Statistical significance was determined by T-Test. P value  $<0.001$ .

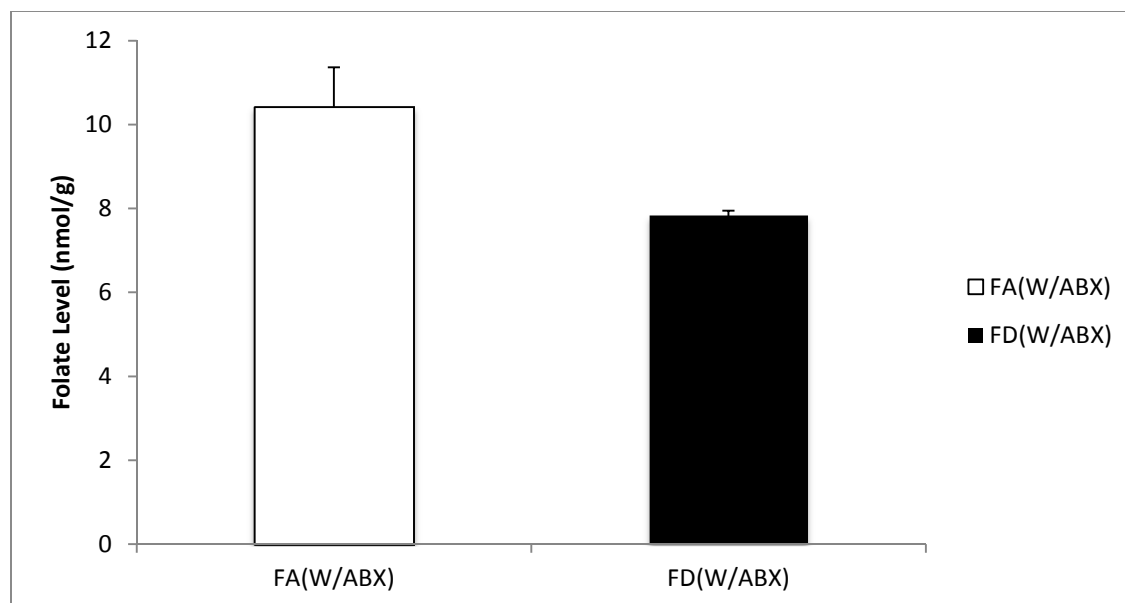


**Figure 29: Effect of Antibiotics on Groups Fed Folate-adequate Diet on small intestine tissue sample.** Comparison between group fed folate-adequate diet without treatment of antibiotics and group fed folate-adequate diet treated with antibiotics. The relative folate level in small intestine tissue was measured using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from DMH group. Statistical significance was determined by T-Test.

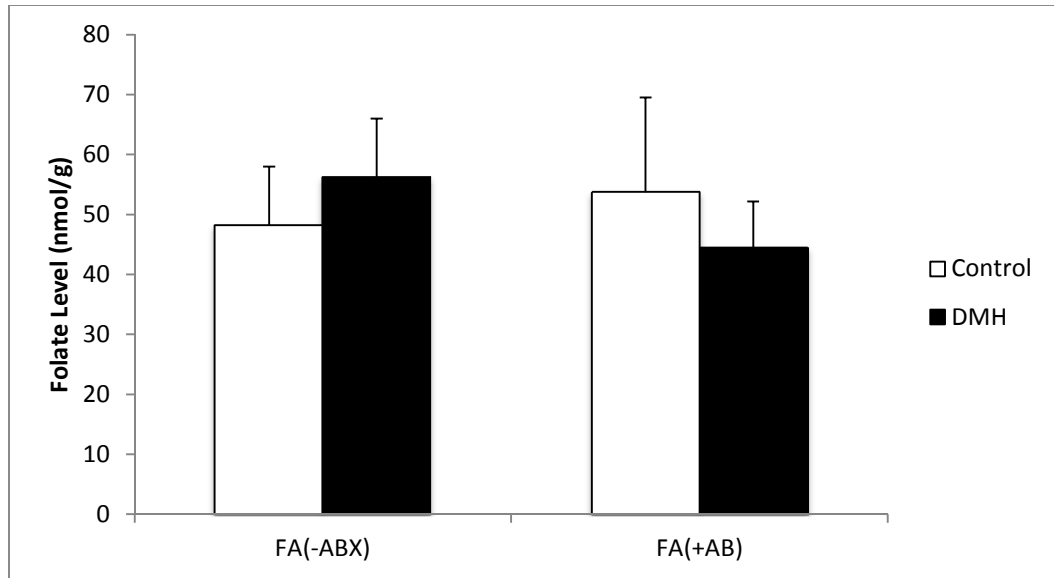


**Figure 30: Effect of Folate Depletion Without Antibiotics on small intestine tissue sample.** Animal group fed folate-adequate diet without using antibiotics compared with animal group fed folate-deficient diet without using antibiotics. Folate level was measured using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from DMH group. Statistical significance was determined by T-Test. P value  $<0.05$ .

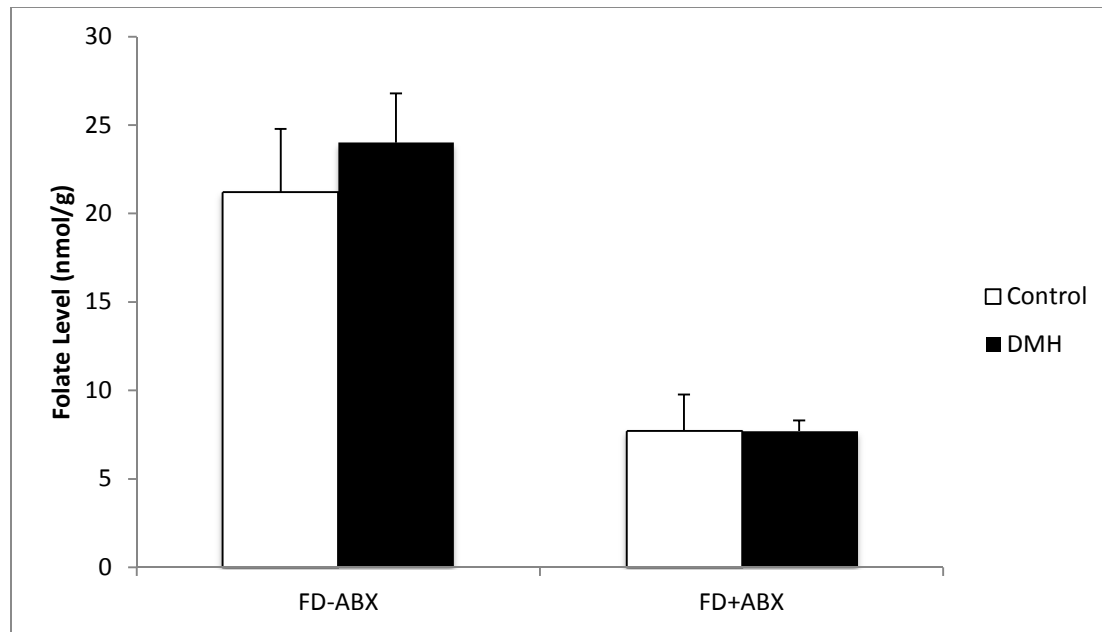




**Figure 31: Effect of Folate Depletion with Antibiotics of small intestine sample.** Comparison between mice groups either fed folate-adequate diet or folate-deficient diet. Folate level was measured using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from DMH samples in both groups. Statistical significance was determined by T-Test. P value  $<0.05$ .



**Figure 32: Effect of DMH on Liver folate levels in Folate adequate experimental groups.** Comparison between two animal groups, both fed folate-adequate diet (2mg/kg, FA). Animals were subjected either to no treatment (control) or treatment with DMH. Group FA (-ABX) were subjected to non-antibiotics treatment. Group FA (+ABX) were subjected to 1 % succinyl sulfathiazole. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from control and DMH groups. Statistical significance was determined by T-Test.



**Figure 33: Effect of DMH on liver folate levels in folate depleted experimental animal groups.** Comparison between two experimental groups fed folate depleted diet (0 mg/kg, FD). Mice were subjected to either no treatment (control) or treatment with DMH. Mice group FD (-ABX) was subjected to non-antibiotic treatment. Mice group FD (+ABX) were subjected to 1% succinyl sulfathiazole. Folate levels were determined using the microbiological assay as described in methods. The data are expressed as means  $\pm$  S.E. For data obtained from control groups and groups treated with DMH. Statistical significance was determined by T-Test. P value for control < 0.05, P value for DMH < 0.01.

Figure 5: Folate level in BNLC2. We have measured folate level in response to depletion of folate from media in BNLC2 cells. Here we find that lack of folate in media preparations results in near total depletion of intracellular folate in the BNLC2 cells, ( $P < 0.001$ ).

Figure 6: Folate level in UNG  $+/+$  and UNG  $-/-$  MEFs in response to folate depletion. From this figure we can see that the MEF cell line is truly depleted in the presence of folate depleted media. The UNG  $-/-$  MEF cells expressed a higher reduction in folate levels compared to UNG  $+/+$  MEF cells. The level is significantly low ( $P < 0.001$ ) in folate. It is determined that folate depleted media exhibited a significant decrease in folate depletion in UNG  $-/-$  MEF cells.

Figure 7: Folate level in UNG  $-/-$  MEF in response to folate depletion. We observed a higher reduction in folate levels in UNG  $-/-$  folate depleted cells compared to UNG  $+/+$  folate added cells. Folate level was significantly low ( $P < 0.001$ ). Results are expected which indicated the absence of folate in growing media induces folate depletion in cells.

Figure 8: FPGS expression in fibroblast and liver cell lines. As described in the introduction, FPGS is responsible for ensuring retention of folate within the cell. We were interested in evaluating the impact of folate deficiency on expression of FPGS, and measured the expression levels as described in Methods. We find that in all cell types, folate depletion results in up regulated expression of FPGS.

Figure 9: Relative GGH gene expression in fibroblast and liver cell lines. GGH gene is essential for determining intracellular folate availability for folate metabolism. As it is mentioned in the introduction, GGH removes glutamyl groups, which allows folate metabolites to leave the cell. We observed in this figure that in all cell line, folate depletion results in down-regulated expression of GGH. The GGH expression is significantly decreased in MEFs cell lines and BNLC2 cells ( $P < 0.001$ ).

Figure 10: Effect of antibiotics on serum folate level. This figure shows the effect of antibiotics on serum folate level between two groups of mice. No significant difference in serum folate level was seen between folate added without ABX and folate added with ABX groups.

Figure 11: The effect of folate deficiency without antibiotics on serum folate level. This figure shows the effect of folate deficiency without antibiotics on serum folate level in control group. A significant decrease in folate depletion was observed in the folate deficient diet without antibiotics mice group when compared to folate added without antibiotics group ( $P < 0.005$ )

Figure 12: The impact of folate deficiency with antibiotics on serum folate level. This figure shows that a folate deficient diet containing no folic acid, along with 1% Succinyl Sulfathiazole, which prevents intestinal production of folate, results in a decrease in serum folate levels. As is shown in this figure, serum folate level decreased significantly ( $P < 0.001$ ) in the folate deficient mice compared to folate adequate mice group.

Figure 13: The effect of DMH on serum folate levels in FA W/0 ABX and FA W/ABX. This figure shows the impact of DMH induction on serum folate level based on the known impact of DMH treatment in the incidence of tumor formation in these animals. Data shows no significant difference between FA (DMH) – ABX mice and FA (DMH) + ABX mice group.

Figure 14: The effect of folate depletion on serum folate levels in mice treated with DMH without antibiotics use. Figure 41 shows that mice fed the folate added diet showed significantly higher ( $P < 0.001$ ) folate levels than mice given folate depleted diet, even when both groups received DMH and no ABX.

Figure 15: The effect of folate depletion on serum folate levels in mice treated with DMH and with antibiotic use. This figure shows that folate added diet with antibiotics results in

significantly higher ( $P < 0.001$ ) serum folate level than folate deficient diet with antibiotics. The folate levels of the deficient animals approached zero.

Figure 16: The effect of DMH on folate serum level without antibiotics. As it is seen from the figure 16, DMH treatment did affect the folate added W/o ABX mice group. Data shows no significant difference between the control group (FA W/o ABX) and DMH treatment group (FA W/o ABX).

Figure 17: The effect of DMH on folate serum level with antibiotics. Figure 4.13 shows that there is no significant difference on serum folate level between control (folate added with antibiotics) and DMH treatment (folate added with antibiotics) mice groups.

Figure 18: Effect of DMH on folate serum level without antibiotics. This figure shows that no significant difference was observed in the control group (FD W/o ABX) as compared to DMH treatment group (FD W/o ABX) on folate serum level.

Figure 19: The effect of folate-depleted diet in DMH treated mice compared to non-DMH treated control group, both groups treated with antibiotics. As is shown in figure 19, serum folate level decreased significantly ( $P < 0.001$ ) in the DMH treatment folate deficient mice as compared to control folate deficient mice. The DMH W/ABX group had serum folate levels lower than the control group. The addition of 1% succinyl sulfathiazole helped in increasing the severity of folate deficiency.

Figure 20: Impact of antibiotics on folate status liver tissue. As it is shown in this figure, we observed the effect of antibiotics on folate status in liver tissue. The mice fed folate added diet with antibiotics show no difference compared to mice fed folate added diet without antibiotics. This result suggests that the addition of antibiotics shows no effect.

Figure 21: The effect of folate depletion without antibiotics on liver tissue folate status. We observed a significant reduction in liver folate concentration in mice fed folate-depleted without antibiotics when compared to mice fed folate-added diet without antibiotics ( $P < 0.005$ ). This shows the effect of folate deficiency and how it increases the depletion level in FD (-ABX) group.

Figure 22: The effect of folate depletion with antibiotics on liver folate tissue sample. Results show the response of folate depletion and antibiotics to liver folate concentration. As shown in this figure, there is a significant decrease in folate-depleted with antibiotics mice as compared to folate-added with antibiotics mice ( $P < 0.05$ ).

Figure 23: The effect of antibiotics on colon tissue level. As shown in this figure, we determined the impact of antibiotics on colon folate level between FA (-ABX) and FA (+ABX) animal model. We observed no significant difference in the level of folate in the control group of colon folate level. Data indicate that there is no decrease in the folate level after eight weeks of antibiotics treatment in FA (+ABX) group as compared to FA (-ABX) group.

Figure 24: The effect of folate depletion without antibiotics on colon tissue level. We observed a significant decrease in the level of folate in FD (-ABX) mice as compared to FA (-ABX) mice. This represents the effect of folate deficiency on colon tissue status. Fig. 24, ( $P < 0.05$ ).

Figure 25: The effect of folate depletion with antibiotics on colon tissue status. We observed a significant decrease ( $P < 0.05$ ) in colon tissue folate status in the folate FD (+ABX) mice compared to FA (+ABX) mice, as shown in Fig. 25.

Figure 26: The effect of antibiotics on kidney folate level. We determined the kidney folate level using the microbiological assay as described in methods. We observed no significant difference in DMH mice fed folate added diet (+ABX) as compared with folate-added diet (-ABX).

Figure 27: The effect of folate depletion without antibiotics on kidney folate level. It is essential to demonstrate that the experimental diet (0 mg/kg folic acid) resulted in decreased kidney folate levels. As it is shown in this figure, a significant decrease in the level of kidney folate level in the folate-deficient W/o ABX mice was observed. In response of folate deficiency in kidney tissue, we observe a significant difference between FA W/o ABX and FD W/o ABX, ( $P < 0.05$ ).

Figure 28: Effect of folate depletion with antibiotics on kidney folate level. As it is shown in this figure, we determined the impact of folate deficiency on kidney tissue sample. We have observed that folate depletion as well as the addition of antibiotics has an impact on kidney tissues. Data shows a significant difference in folate level between folate deficient diet with antibiotics animals and folate added diet with antibiotics animals;  $P < 0.001$ .

Figure 29: Effect of antibiotics on small intestine folate level. We observed no significant differences between folate added diet with the addition of 1 % succinyl sulfathiazole and DMH treatment as compared to folate added without antibiotics and DMH treatment mice groups. Throughout the eight –week feeding study and treatment, no significant difference were observed.

Figure 30: The effect of folate depletion on small intestine folate level. In figure 30, we observed a significant decrease ( $P < 0.05$ ) in small intestine folate level in mice fed folate-depleted diet (-ABX) as to mice fed folate-added diet (W/o ABX).



Figure 31: The effect of folate depletion with antibiotics on small intestine folate status. We have characterized the effect of folate deficiency on small intestine folate level in folate depleted W/ABX mice. The 0 mg/kg folic acid group had folate levels lower than the folate added W/ABX, ( $P < 0.05$ ).

Figure 32: The effect of DMH on liver folate levels in folate adequate experimental group (control and DMH). As it is shown in this figure, data exhibits no significant difference between control and DMH groups of FA (-ABX) and FA (+ABX). Therefore, we conclude that the use of DMH alone does not impact tissue folate status.

Figure 33: The effect of DMH on liver folate levels in folate depleted experimental animal groups. As shown in this figure, we observed no significant difference in the folate deficient mice treated with DMH compared to folate deficient no treatment group (control). Data shows that DMH did not affect the folate level. Since no change observed in the folate level in mice fed folate deficient diet in the addition of 1 % succinyl sulfathiazole as compared to mice fed folate deficient without antibiotics in both control and DMH groups.

## CHAPTER 5

### Discussion and Conclusion

Folate deficiency has been studied mainly due to its association with chronic diseases, such as neural tube defects, megaloblastic anemia, and cancer. Epidemiologic studies suggest that folate status modulates the risk of developing cancers in selected tissues (7). The mechanism of tissue folate status under folate deficiency is shown in this study by observations made in a number of tissues. This study shows the effect of dietary folate on serum and tissue folate status. Folate deficiency will result as a consequence of inadequate folate intake and increased tissue demand. Increased need for folate can happen in various conditions such as pregnancy, anemia, malabsorption, and certain diseases (32).

In the present study we examined the effect of folate deficiency on folate level status in MEFs cell lines and serum, as well as in tissues including liver, kidney, colon and small intestine. For the purpose of this study, four-month old mice were used as the test subject. The mice were given either a folate added or folate deficient diet, which in both cases contained 1% succinylsulfathiazole. This compound is used to inhibit intestinal microflora production of folate, and causes severe folate deficiency. The results from this study demonstrate the up-regulation of mFPGS gene expression and down regulation of GGH gene expression in response to folate depletion. This allows us to connect the gene expression results to folate status. Importantly, in the present study, folate concentrations in serum and tissues were not affected in mice fed folate added diet; however, we observed a severe depletion in mice that were fed a folate-depleted diet. This shows that the combination of folate deficient diet and 1% succinylsulfathiazole leads to increased depletion of folate.

In this study, animals were also injected with dimethylhydrazine (DMH), which is a well-established tissue carcinogen. Our data shows that DMH has no impact on tissue folate level status. Data from previous studies indicated the increased incidence of tumor formation upon DMH treatment when folate is deficient. Based on these findings, in future studies we should pay attention to the impact of DMH on tissues, and test the damage that may be caused by this carcinogen.

As expected in our study, the folate level status changed in the animal model in response to folate deficiency. The folate transporter FPG's gene expression was up-regulated, and the enzyme GGH, which helps to export folate from cells, was down regulated. Finally, we observed that the folate level status shows no significant change when we used the folate-added diet and antibiotics in a young animal model versus an older animal model. This raises a question for further investigation of whether age affects the availability of folate on tissue folate status.

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**ABSTRACT****THE EFFECT OF DIETARY FOLATE ON TISSUE FOLATE STATUS.**

by

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The folates are a family of structurally similar, water soluble, B vitamins that play a crucial role in human health and disease. Folate has an important role in the synthesis of DNA, DNA methylation, and cellular growth. Folate coenzymes function in accepting and donating one-carbon units in the folate metabolic pathway. Folate deficiency impairs DNA synthesis, induces cancer, and causes abnormalities such as neural tube defects and megaloblastic anemia. While scientific research has established the effects of folate deficiency on serum folate, a comprehensive evaluation of the effects of folate deficiency in tissues and cells has yet to be conducted. Using molecular techniques to determine gene expression and a microbiological assay to determine the folate level, we elucidated the mechanism of folate tissue status in response to folate depletion. Our study showed that dietary folate deficiency had an impact on serum folate as well as tissue folate status. This demonstrates the differential effect of folate deficiency on individual health and gene expression.



## **AUTOBIOGRAPHICAL STATEMENT**

### **Education:**

I am graduating in December 2015 with a Master of Science in Nutrition and Food Science from Wayne State University. In 2010, I received a Bachelors in Nutrition Food and Science from Wayne State University, where I also completed an OSHA Laboratory Safety Training Certificate in 2014.. I had come to Wayne State as a transfer student, and had previously attended Henry Ford College and the University of Michigan where I had studied in the Enviornmental Science program. I also hold a degree in Buisness Management from Beirut University College, which I recieved in 1994.

### **Memberships, Volunteer & Professional Work:**

In 2015, I became a member of the American Pharmacy Association as well as the Scientista Organization. I volunteered at United Nations Relief and Works Agency in 1994, and currently coach members of my community who struggle with language. In 2004, I held an internship at Universal Petro Inc.. I established Alpha Holding LLC. in 2009. I was a coordinator at Alpha 3 from 2010 to 2014. Since 2015, I have been working as a coordinator at La Palma Inc.. I also work as a substitute teacher in the Dearborn Public School system through the Professional Educational Services Group.